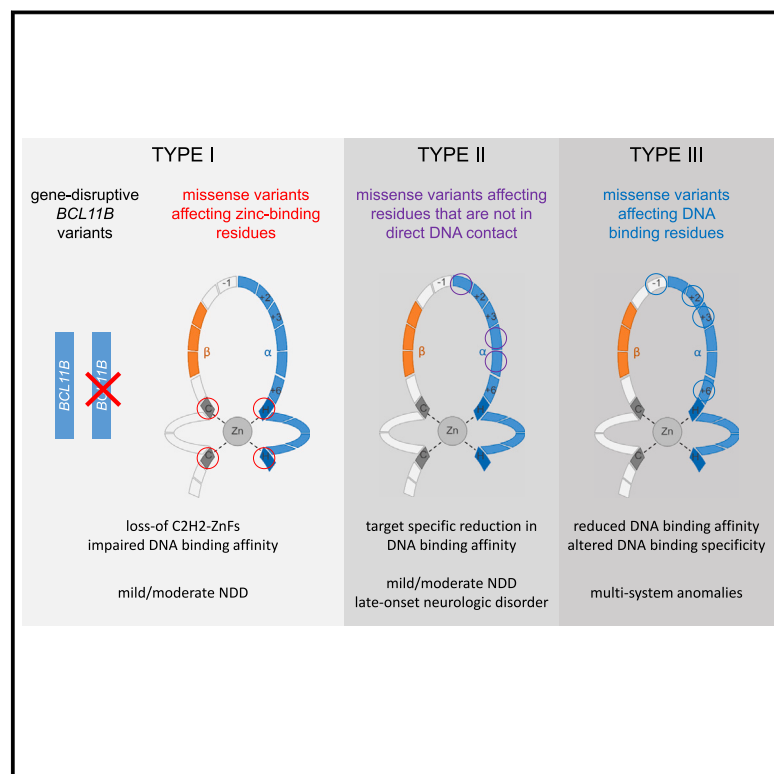


# DNA-binding affinity and specificity determine the phenotypic diversity in *BCL11B*-related disorders

## Graphical abstract



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**Combining genotype-phenotype data of 92 individuals harboring a pathogenic *BCL11B* variant, with immune phenotyping, analysis of chromatin immunoprecipitation DNA-sequencing data, dual-luciferase reporter assays, and molecular modeling, the authors show here that nearby missense variants and different missense variants affecting the identical amino acid do not necessarily cause similar clinical outcomes.**



# DNA-binding affinity and specificity determine the phenotypic diversity in *BCL11B*-related disorders

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## Summary

*BCL11B* is a Cys2-His2 zinc-finger (C2H2-ZnF) domain-containing, DNA-binding, transcription factor with established roles in the development of various organs and tissues, primarily the immune and nervous systems. *BCL11B* germline variants have been associated with a variety of developmental syndromes. However, genotype-phenotype correlations along with pathophysiologic mechanisms of selected variants mostly remain elusive. To dissect these, we performed genotype-phenotype correlations of 92 affected individuals harboring a pathogenic or likely pathogenic *BCL11B* variant, followed by immune phenotyping, analysis of chromatin immunoprecipitation DNA-sequencing data, dual-luciferase reporter assays, and molecular modeling. These integrative analyses enabled us to define three clinical subtypes of *BCL11B*-related disorders. It is likely that gene-disruptive *BCL11B* variants and missense variants affecting zinc-binding cysteine and histidine residues cause mild to moderate neurodevelopmental delay with increased propensity for behavioral and dental anomalies, allergies and asthma, and reduced type 2 innate lymphoid cells. Missense variants within C2H2-ZnF DNA-contacting  $\alpha$  helices cause highly variable clinical presentations ranging from multisystem anomalies with demise in the first years of life to late-onset, hyperkinetic movement disorder with poor fine motor skills. Those not in direct DNA contact cause a milder phenotype through reduced, target-specific transcriptional activity. However, missense variants affecting C2H2-ZnFs, DNA binding, and “specificity residues” impair *BCL11B* transcriptional activity in a target-specific, dominant-negative manner along with aberrant regulation of alternative DNA targets, resulting in more severe and unpredictable clinical outcomes. Taken together, we suggest that the phenotypic severity and variability is largely dependent on the DNA-binding affinity and specificity of altered *BCL11B* proteins.

## Introduction

Proteins containing a Cys2-His2 zinc-finger domain (C2H2-ZnF) constitute the largest class of human tran-

scription factors.<sup>1</sup> They act by either repressing or activating the expression of target genes through binding a specific DNA sequence by their tandem and modular C2H2-ZnFs. Each C2H2-ZnF shares a consensus sequence

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that folds in the presence of zinc (Zn<sup>2+</sup>) to form a two-stranded antiparallel  $\beta$  sheet and a DNA-contacting  $\alpha$  helix. Indeed, ionic bonds between zinc and the two cysteines and two histidines, within each C2H2-ZnF, provide the structural stability of these domains.<sup>2</sup> Each C2H2-ZnF typically contacts three base pairs,<sup>3</sup> whereas the exact DNA binding and sequence preference is mainly regulated by the four “specificity residues” at amino acid positions –1, +2, +3, and +6 within the  $\alpha$  helix.<sup>2,4</sup> In line with their

great abundance, C2H2-ZnF-containing proteins are involved in diverse biological processes including development and differentiation of various organs and tissues.<sup>5,6</sup> Pathogenic variants in genes encoding for C2H2-ZnF-containing proteins are associated with a broad variety of human diseases, including more than 70 associated with a neurodevelopmental or a neurologic disorder.<sup>7</sup>

*BCL11B* (RefSeq NM\_138576.3, MIM: 606558), a member of the Krüppel-like family encoding for B cell

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lymphoma/leukemia 11B, constitutes one example of C2H2-ZnF-containing, DNA-binding transcription factors. As a transcriptional regulator, BCL11B controls various biological processes such as cell death, cell proliferation, and cellular differentiation.<sup>8</sup> Interestingly, due to the specific DNA-binding preferences, the target genes of BCL11B differ among cell types and lineages, thus regulating the gene expression in a context-dependent manner.<sup>9,10</sup> Studies utilizing various genetic manipulations in mice have established pleiotropic functions of Bcl11b in development and differentiation. In the central nervous systems, Bcl11b is involved in the migration, differentiation, and function of neural cells as well as proliferation of neural progenitor cells.<sup>11,12</sup> Bcl11b is necessary for the proper development of the teeth,<sup>13</sup> epidermis,<sup>14</sup> cranium,<sup>15</sup> and white adipose tissue.<sup>16</sup> In the immune system, Bcl11b is involved in the development and maintenance of the T cell lineage<sup>17</sup> and regulation of T cell lineage commitment<sup>18–20</sup> as well as the control of the lineage specification and persistence of type 2 innate lymphoid cells (ILC2s).<sup>21–23</sup> Moreover, mice with a heterozygous loss of *Bcl11b* are susceptible to thymic lymphomas.<sup>24,25</sup> Notably, 16% of human T cell acute lymphoblastic leukemias carry somatic, inactivating *BCL11B* variants,<sup>26,27</sup> whereas activating *BCL11B* genomic rearrangements are potential drivers of lineage-ambiguous leukemias,<sup>28,29</sup> suggesting that BCL11B may act both as a tumor suppressor and an oncogene.

The initial human pathogenic germline *BCL11B* missense variant was identified in a single individual affected with severe combined immunodeficiency (SCID), neurodevelopmental delay, craniofacial abnormalities, absent corpus callosum, and erythematous psoriaform dermatitis (IMD49 [MIM: 617237]).<sup>30</sup> We subsequently established likely gene-disruptive (LGD) germline variants as a cause of a neurodevelopmental delay with impaired T cell development and severe reduction of ILC2s, which did not result in overt clinical signs of immunodeficiency (IDDSFTA [MIM: 618092]).<sup>31</sup> Since then, more than 50 individuals harboring pathogenic or likely pathogenic *BCL11B* variants have been documented.<sup>32–47</sup> DNA methylation analysis of peripheral blood obtained from individuals harboring LGD *BCL11B* variants identified an epismutation of high sensitivity and remarkable specificity,<sup>44</sup> consistent with BCL11B being a transcription factor. However, the molecular mechanisms underlying the associated clinical heterogeneity remain mostly elusive.

Here, we describe 39 previously unreported individuals who harbor heterozygous pathogenic or likely pathogenic *BCL11B* variants and compare their findings to 53 previously documented ones. Integrative analysis of the genotype-phenotype correlations, immune phenotyping, analysis of chromatin immunoprecipitation DNA-sequencing (ChIP-seq) data, dual-luciferase reporter assays, and molecular modeling suggest that, based on the location and nature of the *BCL11B* alteration, the affected individuals can be categorized in at least three clinical subtypes.

## Material and methods

### Human subjects and genetic analyses

Written informed consent for all 39 affected individuals included in this study was obtained from the parents or legal guardians in accordance with protocols approved by the respective ethics committees of the institutions involved in this study. Next-generation-sequencing-based analyses were performed in various independent research or diagnostic laboratories worldwide using previously described procedures,<sup>31,48–52,53</sup> as described in the [supplemental information](#). Classification of the identified variants was based on the American College of Medical Genetics and Genomics (ACMG) guidelines.<sup>54</sup> For all 39 individuals, clinical data and information on genetic testing were uniformly obtained from attending physicians using a structured clinical summary ([supplemental information](#)) and clinical table ([Table S1](#)).

### Immunophenotyping

Processing and analysis of blood samples of individuals harboring a heterozygous *BCL11B* variant followed previously described procedures.<sup>31</sup> In brief, 75  $\mu$ L of whole blood was incubated with combinations of the following fluorochrome-conjugated antibodies for 30 min at room temperature: the “T-regulatory” panel contained anti-CD3 BV510 (clone: OKT3), anti-CD4 AF700 (clone: OKT4), anti-CD8 BV605 (RPA-T8), anti-CD25 BV421 (clone: 96 BC), anti-CD31 APC-Cy7 (clone: WM59), anti-CD39 PE-Cy7 (A1), anti-CD45RA PE-Dazzle (clone: HI100), anti-CD73 PE (clone: AD2), anti-CD127 BV650 (clone: WM59), anti-HLA-DR BV711 (clone: L243), and anti-CCR4 PerCP-Cy-5.5 (clone: TG6); the “T-invariant” panel contained anti-CD3 BV510 (clone: OKT3), anti-CD4 PE-Dazzle (clone: RPA-T4), anti-CD8 AF700 (clone: HIT8a), anti-CD25 BV421 (clone: 96 BC), anti-CD27 BV650 (clone: O323), anti-CD45RO BV785 (clone: UCHL1), anti-CD69 APC-Cy7 (clone: FN50), anti-CD161 BV605 (clone: HP-3G10), anti-CCR6 PerCP-Cy-5.5 (clone: G034E3), anti-CCR7 BV711 (clone: G043H7), anti-Tgd PE-Cy-7 (clone: 11F2), anti-Va7.2 APC (clone: 3C10), anti-Vd1 FITC (clone: TS-1), anti-Vd2 APC (123R3), and anti-Vd9 FITC (IMMU 360); finally, the “T-effector” panel contained anti-CD3 BV785 (clone: OKT3), anti-CD4 APC-Cy-7 (clone: RPA-T4), anti-CD8 BV510 (RPA-T8), anti-CD25 PE (clone: 2A3), anti-CD28 PE-Cy7 (clone: CD28.2), anti-CD38 AF700 (clone: HIT2), anti-CD45RA PE-Dazzle (clone: HI100), anti-CD57 FITC (clone: HCD57), anti-CD95 BV421 (clone: DX2), anti-CD127 BV650 (clone: WM59), anti-CCR7 APC (clone: G043H7), and anti-HLA-DR BV711 (clone: L243). After staining, 1 mL of BD lysing solution was added for lysis of erythrocytes, and stained cells were washed in PBS and resuspended in fluorescence-activated cell sorting (FACS) buffer. Flow-cytometric analysis was performed on an LSR Fortessa (BD Biosciences). Prior to analysis, a spillover spreading matrix was produced, PMT voltages were optimized, and a compensation matrix was calculated. FlowJo software version 10.8.1 (FlowJo, Ashland, OR, USA) was used for manual analysis, using a gating strategy adapted from Sibbertsen et al.<sup>55</sup>

### ChIP-seq analysis

To identify gene targets of BCL11B, we performed extensive analyses of a publicly available ChIP-seq dataset obtained in HEK293 cells (accession ENCODE: ENCSR770PQN), generated by the ENCODE project.<sup>56</sup> Data on the conservative peaks were obtained from the Gene Expression Omnibus (GEO: GSE92041)

bigBed file and ordered by signalValue, i.e., enrichment of signal over input control in the peak. The functional analysis of peaks and gene assignment was performed using the R package ChIPseeker,<sup>57</sup> with default parameters for feature assignment.

### Plasmids

pIRES plasmid containing sequence for full-length Bcl11b wild-type (WT) protein was a kind gift from Prof. Stefan Britsch (Ulm University). To assess BCL11B transcriptional activity, a construct coding for full-length BCL11B WT protein was reinserted from pIRES to pEGFP-N1 by applying restriction digestion with XhoI and AfeI (Thermo Fisher). Digested DNA was separated on 2% agarose gel, and desirable plasmid parts were extracted by a Monarch DNA Gel Extraction Kit (New England Biolabs) following the manufacturer's instructions and ligated with T4 DNA Ligase (New England Biolabs). This, however, produced a STOP codon between protein sequence and EGFP sequence, so additional mutagenesis was performed utilizing a QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies) producing final pEGFP-N1-BCL11B-WT plasmid coding for full-length BCL11B WT protein with C-terminal EGFP tag. pmRFP-N1 and pEGFP-N1-BCL11B-WT were subjected to another restriction digestion with XhoI and HpaI (Thermo Fisher), gel separation, gel extraction, and T4 DNA ligase ligation. Subsequently, the EGFP tag sequence was substituted with mRFP, producing a construct coding for full-length BCL11B WT with a C-terminal mRFP tag, respectively. Missense and frameshift variants were then introduced into the BCL11B WT construct by applying the QuikChange II Site-Directed Mutagenesis Kit with specially designed primers (all primer pairs are available upon request). The correctness of the DNA sequence was verified by direct Sanger sequencing as previously described.<sup>58</sup>

Further, primary reporter plasmids containing distal enhancer-like signature (dELS) sequences within *SHANK3* (MIM: 606230), *LTBP3* (MIM: 602090), and *LTBP4* (MIM: 604710) loci were obtained by amplification with primers (all primer pairs are available upon request) designed to amplify the respective genomic regions from DNA isolated from an unaffected individual. Fresh PCR products were inserted in the pCRTM2.1-TOPO vector (Invitrogen) using a TOPO TA Cloning Kit (Invitrogen) following the manufacturer's instructions. Following restriction digestion with KpnI and XhoI (Thermo Fisher), DNA gel separation, and DNA extraction, inserts were ligated into pGL3-Enhancer vector with a modified coding region for firefly luciferase (Promega). Additionally, pNL1.1.PGK [Nluc/PGK] vector (Promega) was used as a transfection normalization control.

### Luciferase assay

HEK293T cells were transfected 24 h after seeding. A master mix with a primary reporter pGL3-Firefly luciferase plasmid (containing a dELS within either *SHANK3*, *LTBP3*, or *LTBP4*) and pNL1.1.PGK [Nluc/PGK] plasmid was combined in serum-free medium and aliquoted. Each aliquot was then supplemented with either pmRFP-N1 empty control vector, pmRFP-N1-BCL11B-WT, or pmRFP-BCL11B containing one of the selected missense variants. For analyses of potential dominant-negative effect, pEGFP-N1 empty control vector and pEGFP-BCL11B-WT were added in combination. Transfection was carried out for 24 h using Turbofect transfection reagent (Thermo Fisher). Next day, cells were loosened from plates by PBS supplemented with 10 mM EDTA, and transfected cells (RFP-positive, or both RFP- and GFP-positive cells) were sorted on FACS Aria IIIu (Becton Dickinson). Following

sorting, only transfected cells were transferred to white 96-well plates, and the Nano-Glo dual-luciferase reporter assay was carried out following the manufacturer's instructions (Promega). ONE-Glo EX reagent was added to each sample, and the plate was incubated for 10 min at room temperature, shaking on orbital shaker at 300 rpm. Measurement of firefly luminescence activity was performed on a Synergy H1 Microplate reader (BioTek) or a Spark Multimode Microplate Reader (Tecan). An equal volume of NanoDLR Stop & Glo reagent was added to each sample, and the plate was again incubated for 10 min at room temperature, shaking on orbital shaker at 300 rpm, after which Nanoluc activity in the form of luminescence was assessed. Three experiments were carried out for each dELS (at *SHANK3*, *LTBP3*, and *LTBP4* loci) and for each BCL11B missense variant in comparison to WT and empty vector. Each experiment was normalized to the mean luminescence of measured samples to correct for different order of magnitude between experiments. Statistical significance was calculated by one-way ANOVA with Dunnett correction for multiple comparisons (GraphPad Prism).

### DNA-binding motif prediction

The DNA-binding motif landscape and DNA-binding specificity of BCL11B-WT and the missense variants were analyzed using two modeling approaches: "Interactive PWM predictor"<sup>3,59</sup> and "Zinc finger recognition code."<sup>4</sup>

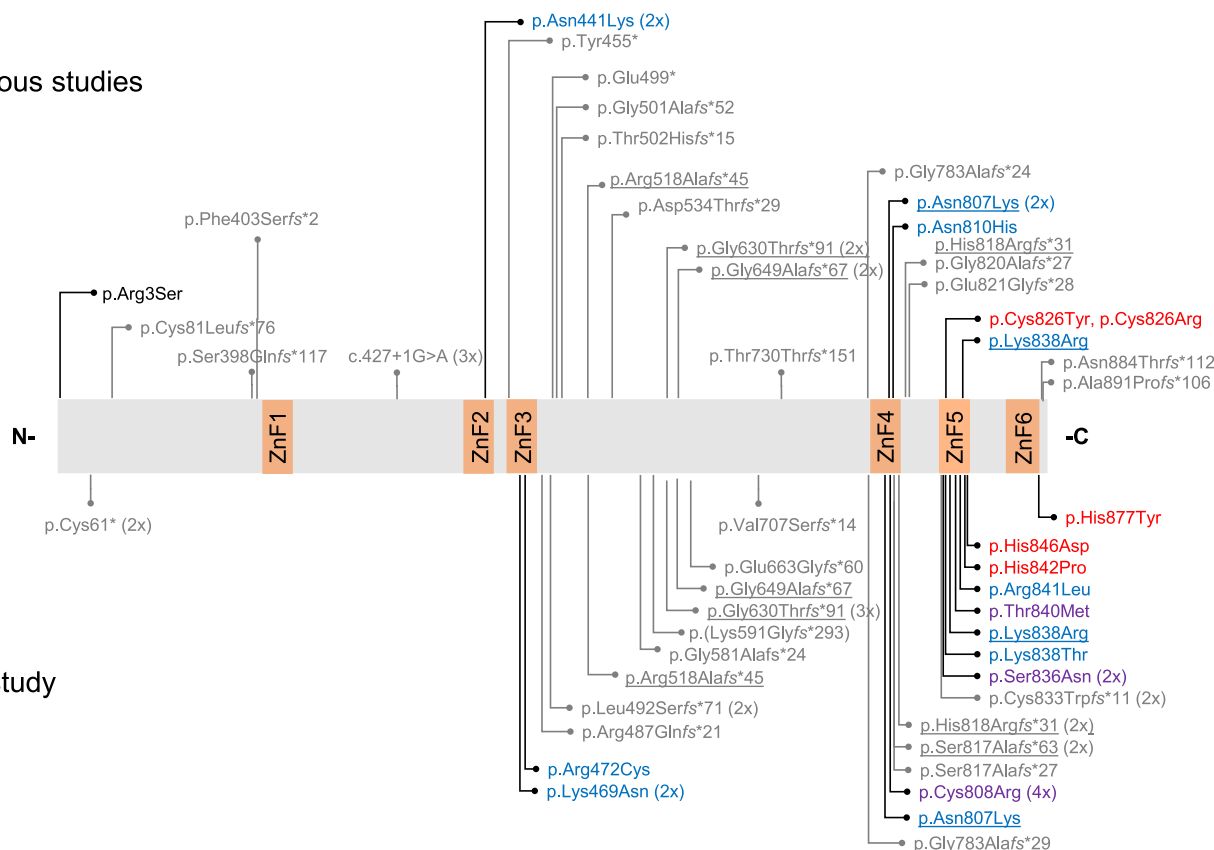
## Results

### Identification of likely gene-disruptive variants in BCL11B

Twenty-six heterozygous, pathogenic, or likely pathogenic BCL11B variants have so far been documented in the literature (Figure 1, upper panel). Here, we report 39 individuals, 30 of whom harbor previously unreported heterozygous variants in BCL11B. Among them, 22 individuals harbor a likely gene-disruptive (LGD) germline variant, out of which 11 have so far not been documented in the literature. Unrelated individuals 1 and 2 harbor an identical *de novo* nonsense variant, NM\_138576.3: c.183\_189delTCAAATG, p.Cys61\*, located in exon 2, predicted to activate the nonsense-mediated mRNA decay and to result in haploinsufficiency. Individuals 3–22 harbor heterozygous frameshift variants located in the last exon (exon 4), which are predicted to escape nonsense-mediated mRNA decay and result in a protein with loss of at least the last two C-terminal DNA-binding zinc-finger domains, C2H2-ZnF5 and C2H2-ZnF6 (Figure 1, lower panel; Table S1). Interestingly, individual 4 harbors a *de novo* c.1474delC, p.Leu492Serfs\*71 variant, which she transmitted to her similarly affected son (individual 5). Siblings, individuals 21 and 22, harbor c.2499delC, p.Cys833Trpfs\*11, which was not identified in any of the parental samples, pointing to a gonadal mosaicism. Moreover, we identified five recurrent frameshift variants: c.1552delC, p.Arg518Alafs\*45, c.1887\_1893delCGGCGGG, p.Gly630Thrfs\*91, c.1944\_1965delCGGCGCGGTCAACGG GCGCGGG, p.Gly649Alafs\*67, c.2448\_2461delGAGCCAC ACCGCGG, p.Ser817Alafs\*63, and c.2439\_2452dupGC ACCGCGGAGCC, p.His818Argfs\*31 (Figure 1). Out of

previous studies

this study



**Figure 1. Locations of identified *BCL11B* germline variants**

Schematic protein structure of *BCL11B*. Previously identified variants are shown in the upper panel. Variants identified in this study are shown in the lower panel. Likely gene-disruptive (LGD) variants are shown in gray. Missense variant in the very N terminus is shown in black. Missense variants affecting “specificity residues” are shown in blue, other missense variants within the  $\alpha$  helix are shown in violet, and missense variants affecting ZnF-contacting cysteines and histidines are shown in red. Recurring variants are underlined. C, C terminus; N, N terminus; ZnF, zinc-finger C2H2 domain.

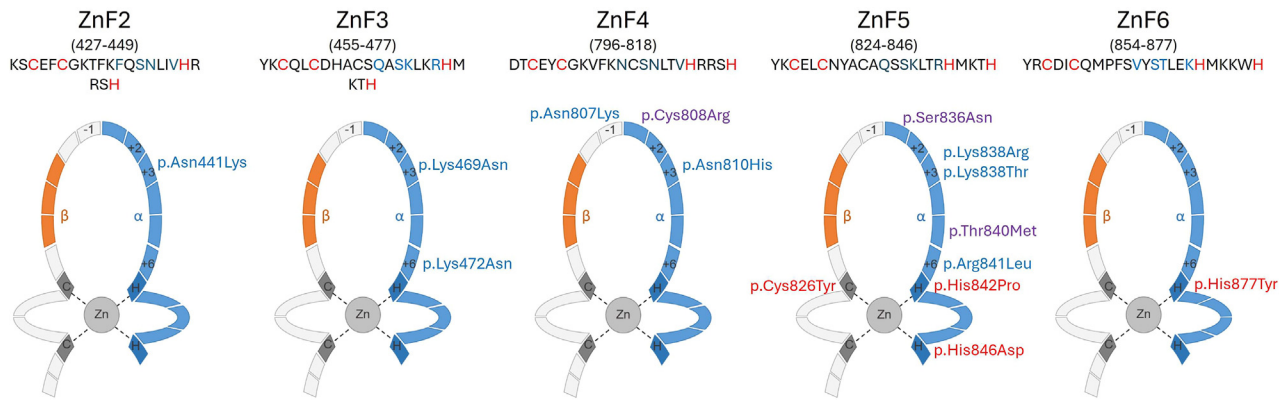
the identified *BCL11B* LGD variants, only the c.1742delG, p.Gly581Alafs\*24, with allele frequency of 0.000003, is present in the gnomAD dataset v.4.1.0<sup>60</sup> (Table S1). *BCL11B*, with a probability of loss-of-function intolerance (pLI) score of 1 and a loss-of-function observed/expected upper bound fraction (LOEUF) score of 0.27, is strongly intolerant to loss-of-function variants. Thus, according to The ACMG guidelines,<sup>54</sup> all variants were classified as either likely pathogenic or pathogenic (Table S1).

#### Identification of *BCL11B* missense variants affecting DNA-binding zinc-finger domains

C2H2-ZnFs harbor a two-stranded antiparallel  $\beta$  sheet and one DNA-contacting  $\alpha$  helix, which regulates DNA binding and sequence preference. The stability of C2H2-ZnFs is provided by the zinc-binding cysteine and histidine residues.<sup>2</sup> Here, we identified 17 individuals harboring a heterozygous amino acid substitution affecting either one of the residues within the DNA-contacting  $\alpha$  helix or one of the zinc-binding residues within one of the *BCL11B*'s C2H2-ZnF. Among them, three individuals harbor a missense variant within C2H2-ZnF3. Individuals 23 and 24 harbor different *de novo* nucleotide substitutions, namely c.1407G>T and

c.1407G>C, respectively that, however, result in identical amino acid substitution p.Lys469Asn. Individual 25 harbors a *de novo* c.1414C>T, p.Arg472Cys variant. Two missense variants were identified within C2H2-ZnF4, one of them previously reported (individual 26, with a *de novo* c.2421C>G, p.Asn807Lys variant).<sup>31,38</sup> Individuals 27–29 inherited a c.2422T>C, p.Cys808Arg from their similarly affected father (individual 30), in whom the variant occurred *de novo*. We found seven missense variants in C2H2-ZnF5. Individuals 31 and 32 harbor the identical *de novo* c.2507G>A, p.Ser836Asn variant. Two individuals harbor an amino acid substitution affecting Lys838, individual 33 harboring a *de novo* c.2513A>C, p.Lys838Thr variant and individual 34 a *de novo* c.2513A>G, p.Lys838Arg variant. Notably, an identical *de novo* p.Lys838Arg variant was recently reported.<sup>35</sup> Further, *de novo* variants c.2519C>T, p.Thr840Met, c.2522G>T, p.Arg841Leu, c.2525A>C, p.His842Pro, and c.2536C>G, p.His846Asp were identified in individuals 35, 36, 37, and 38, respectively. Finally, we identified a single missense variant within C2H2-ZnF6, a *de novo* variant c.2629C>T, p.His877Tyr in individual 39 (Figures 1 and 2).

Notably, six of the identified missense variants within ZnF-C2H2s affected one of the four (–1, +2, +3, and +6)



**Figure 2. Location of missense variants within BCL11B's Cys2-His2 zinc-finger domains**

Schematic diagrams of Cys2-His2 (C2H2-ZnF) zinc-finger domains 2–6. Each C2H2-ZnF contains paired cysteines (C) and histidines (H) that bind the zinc ion (Zn).  $\beta$  sheet is depicted in orange,  $\alpha$  helix is depicted in blue, and 1, +2, +3, and +6 depict “specificity residues” within the  $\alpha$  helix. Missense variants affecting “specificity residues” are shown in blue, other missense variants within the  $\alpha$  helix are shown in violet, and missense variants affecting ZnF-contacting cysteines and histidines are shown in red.

critical C2H2-ZnF “specificity residues”, that is, variants p.Lys469Asn, p.Arg472Cys, p.Asn807Lys, p.Lys838Thr, p.Lys838Arg, and p.Arg841Leu (Figure 2). Such missense variants are thought to impair binding of the altered BCL11B to its physiologic target DNA sites and additionally promote binding to alternative DNA-binding sites.<sup>31</sup> Three missense variants, p.Cys808Arg, p.Ser836Asn, and p.Thr840Met, are localized within ZnF-C2H2s, although not affecting one of the four “specificity residues” (Figure 2). We hypothesized that they may have steric effects on the  $\alpha$  helix containing the DNA-recognition site, thereby reducing the affinity of the altered protein for binding to its target DNA sites. The last three missense variants, p.His842Pro, p.His846Asp, and p.His877Tyr, affect histidine residues that are in direct contact with the zinc cation (Figure 2), likely unfolding C2H2-ZnF5 and ZnF6-C2H2, respectively, and resulting in loss of function.<sup>2</sup>

None of these *BCL11B* missense variants are present in the gnomAD dataset v.4.1.0 (Table S1),<sup>61</sup> indicating that they are extremely rare and likely to be disease associated variants. Thus, according to The ACMG guidelines,<sup>54</sup> again all these variants were classified as either likely pathogenic or pathogenic (Table S1).

### Clinical spectrum of individuals harboring *BCL11B* likely gene-disruptive variants

All individuals reported here harboring an LGD variant are affected by a neurodevelopmental disorder, albeit with variable expressivity and interfamilial variability (Table 1 and Note S1). In more detail, all individuals have mild to moderate intellectual disability and impaired speech development, and most of them (77%) have a delay in motor development. Further common phenotypic features in this cohort include various dental anomalies (Figure 3), such as dental crowding, dental caries, hypodontia, and oligodontia (71%). Additionally, 68% had feeding difficulties in infancy. Interestingly, a review of the literature revealed both dental anomalies (32%) and feeding diffi-

culties (23%) to be less common in previously documented cases (Table 1). Similar to the previous findings, behavioral issues including attention-deficit/hyperactivity disorder (ADHD), autism spectrum disorder, anxiety, obsessive-compulsive disorder, frustration intolerance, and aggressive behavior were relatively common (67%). Additional common phenotypic features included refractive error (45%) and muscular hypotonia (41%). Common facial dysmorphisms (Table 1 and Figure 3) included prominent nose (68%), thin upper lip vermilion (67%), hypertelorism (64%), long philtrum (59%), thin eyebrows (57%), myopathic facial appearance (45%), and small palpebral fissures (32%). Abnormalities of the skull including craniosynostosis, scaphocephaly (sagittal craniosynostosis), microcephaly, macrocephaly, and dolichocephaly were observed in 11 individuals (58%). Compared to previous studies, hypertelorism, skull abnormalities, and myopathic facial appearance were somewhat more common in our case series (Table 1). Regarding the immune system, 12 (55%) had frequent infections in the first years of life, 12 (55%) developed some type of allergy or atopy, and 7 (33%) had asthma, similar to the previous findings (Table 1). Noteworthy, only a single individual (individual 14) developed a hematologic malignancy, a T cell large granular lymphocytic leukemia.

### Clinical variability in individuals harboring *BCL11B* missense variants within C2H2-ZnFs

Compared to the individuals harboring an LGD variant, we observed high clinical variability among individuals harboring a missense variant within the C2H2-ZnFs (Table 2 and Note S1), likely due to variant-specific effects. Individuals 23 and 24, both harboring a *de novo* p.Lys469Asn variant, were affected by neonatal-onset multisystem anomalies. These included common dysmorphic features, sparse hair, global developmental delay (GDD), muscular hypotonia, oral feeding problems, respiratory insufficiency, and early-onset epileptic encephalopathy with

**Table 1. Summary of clinical signs and symptoms of individuals harboring *BCL11B* likely gene-disruptive (LGD) variants**

Clinical findings	Likely gene-disruptive variants (this study)	Likely gene-disruptive variants (previous studies) <sup>31,33,36,38–41,43–47</sup>	Likely gene-disruptive variants (all studies)
Sex	15 female/7 male	22 female/23 male	37 female/30 male
<b>Cognitive and motor development</b>			
Intellectual disability	21/21 (100%)	39/43 (91%)	60/64 (94%)
Speech impairment	22/22 (100%)	41/43 (95%)	63/65 (97%)
Delay in motor development	17/22 (77%)	32/43 (74%)	49/65 (75%)
<b>Dysmorphic features</b>			
Prominent nose	15/22 (68%)	28/44 (64%)	43/66 (65%)
Thin upper lip vermillion	14/21 (67%)	37/44 (84%)	51/65 (78%)
Hypertelorism	14/22 (64%)	17/44 (39%)	31/66 (47%)
Long philtrum	13/22 (59%)	31/44 (70%)	44/66 (67%)
Skull abnormalities	11/19 (58%)	9/33 (27%)	20/52 (38%)
Thin eyebrows	12/21 (57%)	25/44 (47%)	37/65 (57%)
Myopathic facial appearance	10/22 (45%)	10/44 (23%)	20/66 (30%)
Small palpebral fissures	7/22 (32%)	23/44 (52%)	30/66 (45%)
Retrognathia	2/22 (9%)	6/44 (14%)	8/66 (12%)
<b>Neurologic signs</b>			
Behavioral issues	14/21 (67%)	23/42 (55%)	37/63 (59%)
Muscular hypotonia	9/22 (41%)	6/25 (24%)	15/47 (32%)
Reduced fine motor skills	7/22 (32%)	3/16 (19%)	10/38 (26%)
Brain MRI anomalies	3/10 (30%)	10/33 (30%)	13/43 (30%)
Gait abnormalities	5/21 (24%) (1 non-ambulatory)	2/15 (13%)	7/36 (19%)
Seizures	3/22 (14%)	3/40 (8%)	6/62 (10%)
Dystonia	2/22 (9%)	1/14 (7%)	3/36 (8%)
Involuntary movements	1/22 (4.5%)	0/14	1/36 (3%)
Cerebral palsy	1/22 (4.5%)	1/14 (7%)	2/36 (6%)
Hyperkinesia	1/23 (4%)	0/14	1/37 (3%)
<b>Other</b>			
Dental anomalies	15/21 (71%)	14/44 (32%)	29/65 (45%)
Feeding difficulties	15/22 (68%)	5/22 (23%)	20/44 (45%)
Refractive error	10/22 (45%)	13/37 (35%)	23/59 (39%)
Short stature	7/19 (37%)	4/34 (12%)	11/53 (21%)
Clinodactyly	5/22 (23%)	1/14 (7%)	6/36 (17%)
Hypothyroidism	3/22 (14%)	2/32 (6%)	5/54 (9%)
Hair abnormalities	3/22 (14%)	1/41 (2%)	4/63 (6%)
<b>Immune system function</b>			
Frequent infections	12/22 (55%)	22/44 (50%)	34/66 (52%)
Allergies	12/22 (55%)	6/20 (30%)	18/42 (43%)
Asthma	7/21 (33%)	11/38 (29%)	18/59 (31%)
Hematologic malignancy	1/22 (4.5%)	0/42	1/64 (1.5%)



**Figure 3. Images of individuals affected by *BCL11B*-associated disorders**

(A) Dental photos show primary molars of individual 8 immediately after eruption (left). The other two photos were taken at the age of 9 years. The “raised” gums above the left incisor were much more pronounced after the permanent tooth erupted and have slowly but steadily grown well downward.

(B) Images of individual 10 at the age of 5 years shows short forehead, thin eyebrows, “beaked nose” with narrow nasal tip, small mouth, horizontal crease of chin, bilateral earlobe hypoplasia, and clinodactyly of II toe and V finger bilaterally (right panel).

(C) Images of individual 12 at the age of 5 years show a high anterior hairline, small upslanting palpebral fissures, thin eyebrows, a broad forehead, a short nose with anteverted nostrils, a long philtrum, and small mouth with thin lips and downturned corners. Note a somewhat short neck with low posterior hairline.

(D) Images of individual 14 at the age of 19 years show upwardly slanted palpebral fissures, epicanthal folds, broad nasal bridge, long nose, downturned nasal tip, short columella, long philtrum, and thin upper vermillion.

(E) Images of individual 15 at the age of 12 years show a high anterior hairline, a long nose with protruding columella hypoplastic alae nasi, thin upper lip vermillion, and low-set ears.

(F) Images of individual 18 at the age of 3 years show midface flattening, flattened nasal bridge with rounded nasal tip, micrognathia, and a small mouth.

(G) Images of individual 23 at the age of 20 months show a high anterior hairline, white and thin hair, absent eyelashes, small mouth, and micrognathia.

agenesis or absence of corpus callosum. Abnormalities of the skin included pigmentary mosaicism in individual 23 (Figure 3E) and periorbital skin and scalp eczema in individual 24. Both had an idiopathic CD4<sup>+</sup> T cell lymphocytopenia (ICL) and died following infections in the third and second year of life, respectively. Individual 25, who harbors a *de novo* p.Arg472Cys variant, had developmental

and epileptic encephalopathy with global neurologic regression. Individual 26 had GDD but did not develop congenital erosive dermatitis, which we previously observed in an individual harboring the identical *de novo* p.Asn807Lys variant.<sup>31</sup>

The four affected family members harboring the p.Cys808Arg variant (individuals 27–30) showed interfamilial clinical variability. Twins (individuals 27 and 28) exhibited moderate delay in speech and motor development, poor fine motor skills, hyperkinetic movement disorder, anxiety, and obsessive-compulsive disorder. Their younger brother (individual 29) had an articulation disorder, severe rigidity dystonia, and ataxic gait. Their father (individual 30) had poor fine motor skills and mild hyperkinetic movement disorder, and developed Henoch-Schonlein purpura in

**Table 2. Clinical signs and symptoms of individuals harboring pathogenic *BCL11B* missense variants within Cys2-His2 zinc-finger domains (C2H2-ZnF)**

Location of <i>BCL11B</i> missense variants	Affecting a “specificity residue” within a C2H2-ZnF						Within a C2H2-ZnF but not affecting a “specificity residue”				Within a C2H2-ZnF, affecting a zinc-binding cysteine or histidine residue		
	Asn441Lys	Lys469Asn	Arg472Cys	Asn807Lys1	Asn810His	Lys838Thr	Lys838Arg1	Arg841Leu	Cys808Arg	Ser836Asn	Thr840Met	Cys826Tyr	His842Pro
Exact <i>BCL11B</i> missense variants													
			this study	this study		this study	this study	this study	this study	this study	this study	Cys826Arg	His846Asp
													His877Tyr
													this study
Sex	2M <sup>30,32</sup>	1F/1M	1F	2F <sup>31,38</sup> /1M <sup>31,38</sup>	1F <sup>36</sup>	1M	1F <sup>35</sup> /1M	1M	4M	1F/1M	1M	1F <sup>37</sup> /1M <sup>44</sup>	1F/2M
<b>Cognitive and motor development</b>													
Intellectual disability	1/1	2/2	+	2/2	+	+	2/2	+	0/4	1/2	+	2/2	3/3
Speech impairment	1/1	2/2	+	2/2	+	+	2/2	+	3/4	2/2	–	1/1	3/3
Delay in motor development	1/1	2/2	+	3/3	+	+	2/2	+	2/4	2/2	–	1/1	3/3
<b>Dysmorphic features</b>													
Prominent nose	2/2	0/2	–	1/3	+	+	0/2	+	0/4	2/2	+	1/1	0/3
Thin upper lip vermillion	0/1	2/2	+	2/3	+	+	1/2	+	3/4	2/2	–	1/1	2/3
Hypertelorism	1/2	1/2	+	2/3	+	–	1/2	+	0/4	2/2	–	0/1	1/3
Long philtrum	2/2	0/2	+	1/3	+	+	1/2	+	2/4	1/2	–	0/1	1/3
Skull abnormalities	2/2	2/2	+	1/3	–	+	0/2	–	0/4	2/2	+	1/2	2/3
Thin eyebrows	2/2	0/2	+	0/3	+	–	0/2	–	0/4	0/2	+	1/1	0/3
Myopathic facial appearance	0/2	0/2	–	2/3	+	+	2/2	+	0/4	0/2	–	0/1	1/3
Small palpebral fissures	2/2	0/2	–	2/3	+	–	0/2	+	0/4	1/2	–	0/1	1/3
Retrognathia	2/2	2/2	+	0/3	–	–	0/2	+	0/4	1/2	+	N/A	0/3
<b>Neurologic signs</b>													
Behavioral issues	?	?	?	0/2	?	?	0/2	+	2/4	0/2	+	0/1	3/3
Muscular hypotonia	2/2	2/2	+	2/2	–	+	2/2	+	3/4	2/2	–	N/A	1/3
Reduced fine motor skills	?	?	+	0/2	N/A	N/A	N/A	–	3/4	0/2	+	N/A	0/3
Brain MRI anomalies	2/2	2/2	+	1/2	N/A	+	1/1	N/A	1/1	0/2	–	0/2	2/3
Gait abnormalities	1/1	?	+	2/3	+	+	2/2	–	4/4	2/2	–	N/A	0/3
Seizures	1/1	2/2	+	0/2	N/A	+	0/2	–	0/4	0/2	–	0/1	0/3
Dystonia	1/1	?	?	0/2	N/A	–	2/2	–	3/4	0/2	–	N/A	0/3
Involuntary movements	N/A	?	+	0/2	N/A	–	2/2	–	0/4	0/2	+	N/A	0/3

(Continued on next page)

**Table 2. Continued**

Location of <i>BCL11B</i> missense variants	Affecting a “specificity residue” within a C2H2-ZnF								Within a C2H2-ZnF but not affecting a “specificity residue”			Within a C2H2-ZnF, affecting a zinc-binding cysteine or histidine residue	
Cerebral palsy	N/A	?	+	1/3	N/A	+	2/2	–	0/4	0/2	–	N/A	0/3
Hyperkinesia	N/A	?	N/A	0/2	N/A	N/A	N/A	–	3/4	0/2	–	N/A	0/3
<b>Other</b>													
Feeding difficulties	1/1	2/2	–	2/3	+	+	1/2	–	1/4	2/2	–	1/1	2/3
Dental anomalies	1/2	1/2	–	3/3	+	–	0/2	N/A	2/4	1/2	–	0/1	1/3
Refractive error	1/1	1/1	–	0/3	N/A	+	0/2	–	0/4	0/2	–	0/1	2/3
Short stature	N/A	1/1	–	0/2	N/A	+	1/1	N/A	0/4	0/2	–	N/A	1/3
Clinodactyly	N/A	0/2	–	0/3	N/A	–	0/2	–	0/4	0/2	–	N/A	0/3
Hypothyroidism	N/A	0/2	–	0/3	N/A	–	0/2	–	0/4	0/2	–	0/1	0/3
Hair abnormalities	N/A	2/2 (sparse)	–	0/3	–	–	0/2	–	0/4	0/2	–	1/1	0/3
<b>Immune system function</b>													
Immune response anomalies	1 SCID	2/2	–	1/3	SCID	SCID	2/2	+	4/4	2/2	–	1/2	1/3
	1 ICL	ICL					(1 ITP)		FI	FI		FI	FI
Allergies	N/A	1/1	–	1/3	N/A	–	0/2	+	0/4	0/2	–	1/2	2/3
Asthma	N/A	0/1	–	1/3	N/A	–	0/2	+	0/4	1/2	–	2/2	0/3
Hematologic malignancy	N/A	0/2	–	0/3	N/A	–	0/2	–	0/4	1/2	–	NA	0/3

F, female; M, male; +, present; –, absent; ?, too young to evaluate; N/A, unknown; SCID, severe combined immunodeficiency; ICL, idiopathic CD4<sup>+</sup> T cell lymphocytopenia; ITP, idiopathic thrombocytopenic purpura; FI, frequent infections.

adulthood. All family members suffered from frequent viral infections and/or environmental allergies (Table 2 and Note S1). Individuals 31 and 32 harbor the identical *de novo* p.Ser836Asn variant, had GDD, and had a history of frequent infections. Notably, individual 32 developed acute T cell lymphoblastic leukemia at the age of 12 years. Individual 33, harboring a *de novo* p.Lys838Thr variant, had severe GDD with focal seizures, dysgenesis of the corpus callosum, cortical vision loss, and SCID. Individual 34, harboring a heterozygous p.Lys838Arg variant, was affected with severe GDD, was non-ambulatory, and had no speech at the age of 24 years, with spastic cerebral palsy, abnormal involuntary movements, thrombocytopenia purpura, and a history of frequent infections. Following an unremarkable early development, individual 35, who harbors a *de novo* p.Thr840Met variant, starting from the age of 4 years developed mild intellectual disability, developmental coordination disorder, ADHD, and subcortical myoclonias. Individual 36, harboring a *de novo* p.Arg841Leu variant, had GDD, autistic features, food allergies, asthma, and a mild decrease of T cell counts. Individual 37, harboring a *de novo* p.His842Pro variant, was affected by GDD, lingual dyspraxia, and asthma. Individual 38, harboring a *de novo* p.His846Asp variant, had GDD with autistic features, food allergies, and asthma. Lastly, individual 39, harboring a *de novo* p.His877Tyr variant, was affected by GDD with autistic features. Notably, all individuals harboring a missense variant displayed variable dysmorphic features, as summarized in Table 2.

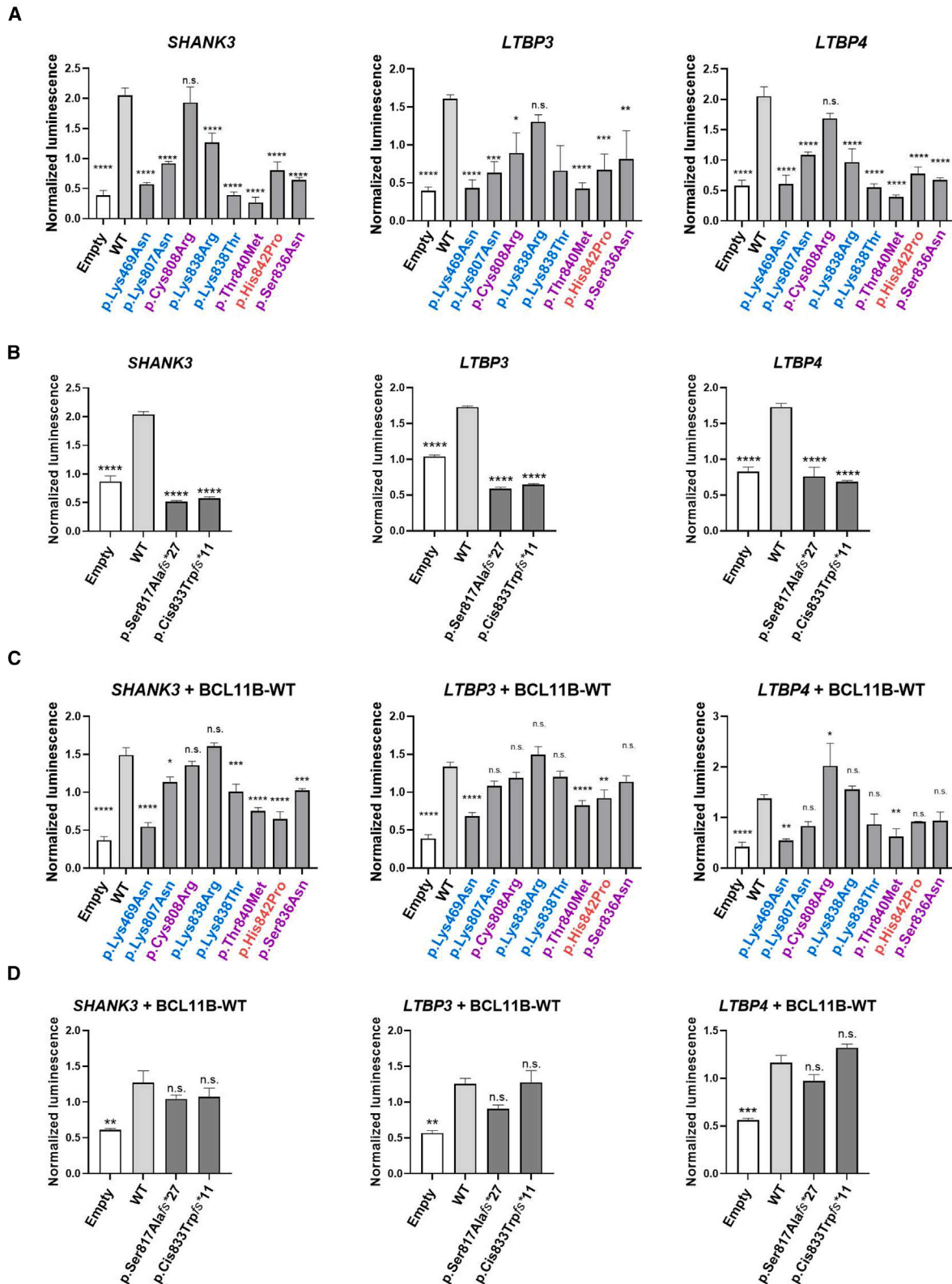
#### Common immune traits in individuals with *BCL11B* variants

Analyses in mice have established Bcl11b as essential for the development of T cells and the specification and maintenance of ILC2s.<sup>17–23</sup> In line with murine data, we have previously observed alterations in the T cell compartment and severe reduction of ILC2s in individuals harboring pathogenic, mostly LGD, *BCL11B* variants.<sup>31</sup> Thus, to further delineate the *BCL11B*-associated immune phenotype, we analyzed the immune compartment of 13 available affected individuals included in this study. Six individuals (individuals 2, 6, 8, 9, 14, and 15) harbored an LGD variant, and seven (individuals 25, 27, 28, 29, 31, 32, and 35) harbored a missense variant. Similar to our previous findings, leukocyte counts were mostly within the normal range (data not shown). Furthermore, six out of nine individuals analyzed had high eosinophil counts, in line with increased prevalence of allergy/atopy (Table S2). The absolute count and frequency of T cells was within the normal range in all individuals, except individual 31 who had few T cells, and the two cases with malignancies (individuals 13 and 32) with aberrant lymphocyte cell compartment due to the tumor (T cell large granular lymphocytic) and chemotherapy, respectively. The frequency of B and natural killer cells was also within normal range, except in individuals with a malignancy (individuals 14 and 32). Detailed anal-

ysis of the T cell compartment revealed an over-representation of cells bearing the T- $\gamma\delta$  receptor in 10 out of 12 individuals without a malignancy and an abnormally low percentage of CD4<sup>+</sup> recent thymic emigrants (RTEs), that is, the cells that have recently migrated from the thymus to the peripheral blood, indicating impaired T- $\alpha\beta$  cell development, in nine individuals (Table S2). As a result, most of the individuals with few RTEs also had a low frequency of naive CD4<sup>+</sup> cells and CD8<sup>+</sup> T cells and a concomitant higher frequency of memory T cells (data not shown). Interestingly, although we previously suggested that the reduction of ILC2s is pathognomonic for *BCL11B*-associated disorders, in this cohort we observed a low proportion of ILC2s in only eight individuals. Of note, the reduction in the frequency of ILC2s occurred in all analyzed individuals harboring an LGD *BCL11B* variant and both individuals harboring p.Ser836Asn. In contrast, family members (individuals 27–29) who harbor p.Cys808Arg, and individuals 25 and 35 who harbor p.Arg472Cys or p.Thr840Met, respectively, had ILC2s within the normal range (Table S2). Taken together, our data show that T cell development is affected in nearly all analyzed individuals and indicate low thymic output and a bias in the usage of the T cell receptor chains. In contrast, the ILC2 compartment seems to be altered in a variant-specific manner, primarily in individuals harboring LGD variants.

#### *BCL11B* ZnF-C2H2 missense variants differentially affect binding to target genes

To further confirm the pathogenicity of the here identified ZnF-C2H2 missense variants and possibly provide an explanation for the variability in clinical and immune phenotype, we next analyzed the publicly available ChIP-seq (ChIP with massively parallel DNA sequencing) dataset obtained in HEK293 cells (accession ENCODE: ENCSTR770PQN) generated by the ENCODE project.<sup>56</sup> Using the ChIPseeker package,<sup>57</sup> we annotated 9,009 conservative peaks. The full list including annotation with peaks ordered by descending signal value (i.e., enrichment of signal over input control) can be found in Table S3. Notably, almost half of the peaks, 4,123 (46%), were located within 1 kb each side of an annotated transcription start site (TSS) (Figure S1). To analyze whether the ZnF\_C2H2 missense variants affect the DNA-binding affinity, we chose three putative binding targets, each affecting a candidate *cis*-regulatory element (cCRE),<sup>62</sup> namely, a dELS within *SHANK3* (ENCODE: EH38E2173377), a dELS within *LTBP3* (ENCODE: EH38E1545299), and a dELS within *LTBP4* (ENCODE: EH38E1954213) (Figure S2). Significantly stronger luciferase activity for the three target dELS in HEK293T cells transfected with a plasmid encoding WT human BCL11B compared to those transfected with the empty vector confirmed the binding to all three targets (Figure 4A). We next measured the transcriptional activity of the three missense variants within a C2H2-ZnF that do not affect a “specificity residue,” p.Cys808Arg, p.Ser836Asn, and p.Thr840Met, and of four missense



**Figure 4. Analysis of DNA-binding affinity of selected *BCL11B* missense variants**

Luciferase assay utilizing HEK293T cells.

(A) Firefly luciferase plasmid (containing a dELS within either *SHANK3*, *LTBP3*, or *LTBP4* locus) and pNL1.1.PGK [Luc/PGK] plasmid were transfected together with empty pRFP-N1, pRFP-N1-*BCL11B*-WT, or one of the depicted missense variants in pRFP-N1-*BCL11B* plasmid. Results are presented as the means from three independent experiments. Each experiment was normalized to the mean luminescence of measured samples to correct for different order of magnitude between experiments.

(B) Experiments as in (A) performed for selected frameshift variants.

(legend continued on next page)

variants affecting a “specificity residue” within a C2H2-ZnF, p.Lys469Asn, p.Asn807Lys, p.Lys838Thr, and p.Lys838Arg along with p.His842Pro, affecting a zinc-binding histidine residue within C2H2\_ZnF5. For the analysis of the p.Lys469Asn variant, we introduced only the nucleotide substitution c.1407G>T as identified in individual 23. The measurement of the luciferase activity revealed that all mutant proteins, apart from p.Ser836Asn and p.Cys808Arg, displayed significantly reduced activation for each of the analyzed targets compared to the WT. The p.Ser836Asn showed decreased activation at the *SHANK3* and *LTBP4* loci, while p.Cys808Arg only showed this at the *LTBP3* locus (Figure 4A). To compare the findings obtained by analyzing missense variants to proteins lacking C-terminal C2H2-ZnFs, we next analyzed two frameshift variants that were identified in affected individuals. p.Ser817Alafs\*27, which alters the amino acid sequence of the C2H2-ZnF4, likely results in a truncated protein lacking the last two C2H2-ZnFs, and p.Cys833Trpfs\*11, which alters the amino acid sequence of C2H2-ZnF5, likely results in a truncated protein lacking the last C2H2-ZnF6. Notably, both frameshift variants, to a similar degree, displayed significantly reduced activation for each of the analyzed targets compared to the WT (Figure 4B).

We next assessed whether any of the missense variants exert a dominant-negative effect. When each of the mutants was co-transfected with the WT *BCL11B* vector, we observed differing results. Co-expression of p.Cys808Arg and p.Ser836Asn did not inhibit WT activation for any of the three targets. Mutants p.Asn807Lys, p.Lys838Arg, and p.His842Pro inhibited WT activation only at the *SHANK3* locus, and p.Thr840Met inhibited WT activation at *SHANK3* and *LTBP3* loci, whereas p.Lys469Asn and p.Lys838Thr inhibited WT activation for all three targets, albeit to a different degree. Thus, these data suggest that specific ZnF-C2H2 missense variants exert a dominant-negative effect in a target-dependent manner (Figure 4C). To further evaluate whether this dominant-negative effect is specific for missense variants, we repeated the experiments analyzing the two frameshift variants, p.Ser817Alafs\*27 and p.Cys833Trpfs\*11. Notably, their co-expression did not inhibit WT activation for any of the three targets, suggesting that they do not exert a dominant-negative effect (Figure 4D).

#### Missense *BCL11B* variants affecting ZnF-C2H2 specificity residues are predicted to regulate alternative genome regions

Transcriptional regulation governed by C2H2-ZnF-containing proteins is not only dependent on DNA-binding affinity but also on their specificity. The latter is mainly

controlled by the four “specificity residues” that are the most exposed amino acids on the surface of the DNA-contacting  $\alpha$  helix.<sup>2</sup> We have previously postulated that *BCL11B* missense variants affecting a “specificity residue” within a C2H2-ZnF may cause clinical heterogeneity through acquisition of alternative DNA-binding regions.<sup>31</sup> Accordingly, the affected individuals presented here who harbor a *BCL11B* missense variant affecting a “specificity residue” displayed a broader and more severe phenotype compared to other individuals harboring *BCL11B* variants. However, this clinical variability could not be explained by differential binding affinity in our assays (Figure 4). We therefore utilized two independent bioinformatics algorithms, “Zinc Finger Recognition Code”<sup>4</sup> and “Interactive PWM predictor,”<sup>3,59</sup> to analyze the DNA-binding preferences of the *BCL11B* missense variants within C2H2-ZnFs. Importantly, none of the other C2H2-ZnF missense variants within the DNA-contacting  $\alpha$  helix were predicted to regulate alternative targets, suggesting that their main mode of action is the reduced binding capacity due to steric effects on the  $\alpha$ -helix-containing DNA-recognition site. In contrast, both modeling approaches, albeit in a different manner, suggest that all missense variants affecting a “specificity residue” result in binding to different alternative genomic sequences, thereby probably regulating different alternative target genes (Figures 5 and S3) and providing further clarification of the observed clinical heterogeneity.

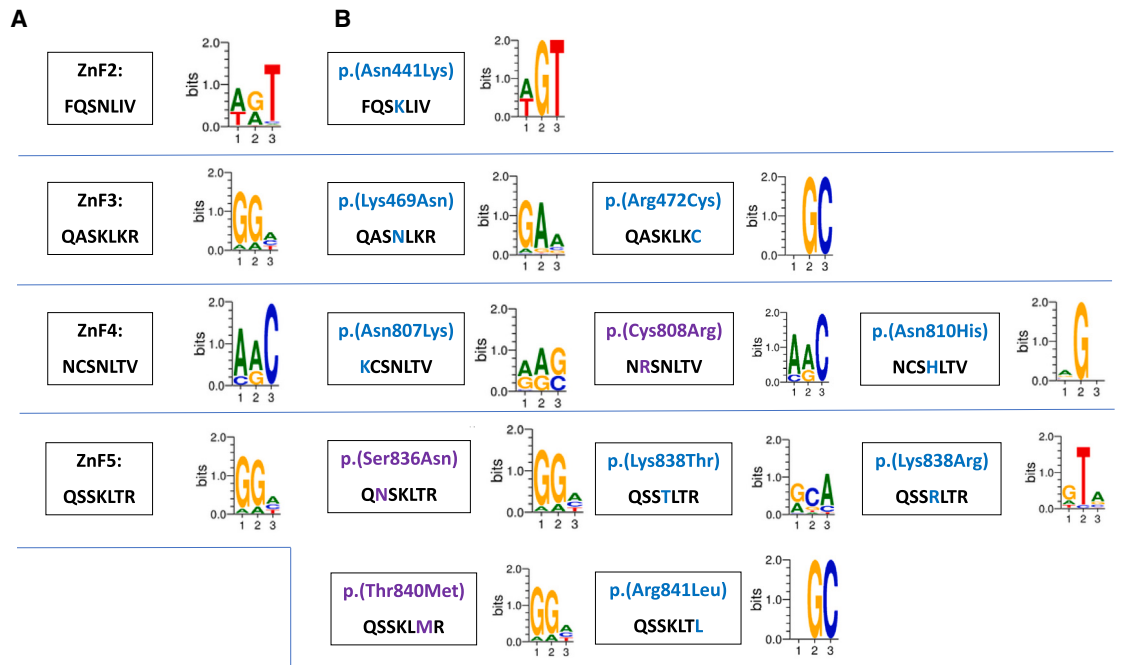
## Discussion

This study further delineates the clinical spectrum of *BCL11B*-related neurodevelopmental disorders through analysis of 92 affected individuals, 39 of whom have so far not been reported in the literature. All affected individuals display impaired function of the nervous system, ranging from severe GDD to poor fine motor skills and late-onset, mild hyperkinetic movement disorder. Most individuals display impaired development of the immune system, although overt signs of immunodeficiency are observed exclusively in individuals harboring missense variants affecting “specificity residues” within the DNA-contacting  $\alpha$  helices. In line with previous findings in mice,<sup>13–15</sup> further commonly affected organs include the cranium, skin, and teeth. Based on the clinical, genetic, and molecular findings, we propose that *BCL11B* alterations result in a phenotypic spectrum that can be classified into at least three subtypes.

Individuals harboring heterozygous LGD *BCL11B* variants are affected by mild to moderate intellectual disability and impaired speech development, and the majority

(C) For analyses of potential dominant-negative effect, pEGFP-*BCL11B*-WT was added in combination with the above. Statistical significance was calculated by one-way ANOVA with recommended Dunnett correction for multiple comparisons (GraphPad Prism). Means  $\pm$  standard deviation values are based on three independent experiments. Significantly different from *BCL11B*-WT: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ; n.s., not significantly different from *BCL11B*-WT.

(D) Experiments as in (C) performed for selected frameshift variants.



**Figure 5. Modeling of BCL11B DNA-binding specificity due to BCL11B missense variants**

(A) Predicted DNA-binding sites of the BCL11B-WT for the four C2H2-ZnFs in which missense variants were identified.

(B) Predicted DNA-binding sites for each of the missense variants within the  $\alpha$  helix. Note that all missense variants affecting a “specificity residue” (shown in blue) alter the DNA-binding site and are therefore predicted to bind to different alternative genomic sequences as compared to the BCL11B-WT. Missense variants within the  $\alpha$  helix not affecting a “specificity residue” (shown in violet) do not alter the DNA-binding specificity. Data are based on “Interactive PWM Predictor.”<sup>3</sup>

display a delay in motor development. Further common clinical signs and symptoms include feeding difficulties in infancy, various dental anomalies (dental crowding, dental caries, hypodontia, and oligodontia), behavioral issues (ADHD, autism spectrum disorder, anxiety, obsessive-compulsive disorder, frustration intolerance, and aggressive behavior), refractive error, muscular hypotonia, and common facial dysmorphisms. Regarding the function of the immune system, these individuals show impaired T cell development, with low numbers of RTEs, and low frequencies of ILC2s. They commonly suffer from frequent infections (~50%) and have an increased risk of developing asthma and allergies (~40%, Tables 1 and S2). In addition, distinct abnormalities of the skull were found in 38% of affected individuals, including craniosynostosis, scaphocephaly, microcephaly, macrocephaly, and dolichocephaly. Moreover, we document the occurrence of short stature, clinodactyly, retrognathia, and hypothyroidism (Table 1). These LGD variants result in either haploinsufficiency or truncated proteins with loss of at least the last two C-terminal DNA-binding zinc-finger domains (C2H2-ZnFs). *In vitro* analyses, using two exemplary frameshift variants, p.Ser817Alafs\*27 and p.Cys833Trpfs\*11, have shown that both result in reduced DNA-binding affinity without exerting a dominant-negative effect on the BCL11B targets analyzed in this study.

Individuals harboring a heterozygous BCL11B missense variant affecting a zinc-binding residue within C2H2-

ZnFs mostly display clinical similarities to the individuals harboring LGD variants. Individuals 37–39, harboring p.His842Pro, p.His846Asp, or p.His877Tyr, respectively, were affected by GDD with behavioral issues. Two had hyperopia and feeding difficulties in early childhood. Although we were unable to obtain fresh blood samples for immune phenotyping, two were reported to have eosinophilia, asthma, and allergies. Similar clinical presentation, a neurodevelopmental delay with asthma, and a primary atopic disorder were reported in two individuals harboring *de novo* missense variants that affect a zinc-binding cysteine residue within C2H2\_ ZnF5, p.Cys826Arg and p.Cys826Tyr.<sup>44</sup> Our *in vitro* analyses, using the exemplary variant p.His842Pro, suggested that these missense variants lead to reduced DNA-binding affinity and may additionally exert a dominant-negative effect in a target-dependent manner, therefore confirming the importance of zinc coordination for the proper folding and stability of the C2H2-ZnFs.<sup>2</sup>

The phenotypic variability is even more pronounced in individuals harboring missense variants affecting the DNA-contacting  $\alpha$  helix within a C2H2-ZnF that affect neither a zinc-binding residue nor a “specificity residue.” We observed interfamilial phenotypic variability in individuals 27–30, all harboring the p.Cys808Arg variant. Twins (individuals 27 and 28) presented with moderate speech and motor developmental delay, poor fine motor skills, hyperkinetic movement disorder, and behavioral

issues, while their father (individual 30) had poor fine motor skills and late-onset, mild hyperkinetic movement disorder. All members of the family had an intact ILC2 compartment, and the changes in the T cell compartment were rather mild, except in individual 28, who had a very high frequency of  $\gamma\delta$ -T cells. Both individuals (individuals 31 and 32) harboring the *de novo* p.Ser836Asn variant displayed many clinical similarities to the individuals harboring LGD variants. Individual 32, however, developed acute T cell lymphoblastic leukemia, while individual 31, at age 2 years, does not have a malignancy but has a profoundly affected T cell compartment and reduced ILC2s, similar to individuals harboring LGD variants. Following unremarkable early development, individual 35, harboring a *de novo* p.Thr840Met variant, developed mild intellectual disability, developmental coordination disorder, ADHD, and subcortical myoclonias at the age of 4 years. This individual had a minor alteration in the T cell compartment, with slightly high frequency of  $\gamma\delta$ -T cells. Notably, reduced DNA-binding affinity for p.Cys808Arg was observed only at the *LTBP3* locus and for p.Ser836Asn at *SHANK3* and *LTBP3* loci. Contrarily, p.Thr840Met displayed impaired DNA-binding affinity at all three analyzed loci and seems to exert a dominant-negative effect on *SHANK3* and *LTBP3* loci. These findings suggest that observed phenotypic and immune variability results from the impaired DNA-binding affinity in a locus-specific and dosage-dependent manner.

Finally, the *BCL11B* subtype characterized by the most severe clinical outcome and largest clinical heterogeneity is attributable to the missense variants affecting one of the four “specificity residues” within C2H2-ZnFs. Indeed, such missense variants result in multisystem anomalies with SCID<sup>30</sup> or syndromic ICL leading to early demise due to infections (individuals 23 and 24; p.Lys469Asn) but also in isolated, severe epileptic encephalopathy (individual 25; p.Arg472Cys). In addition, somewhat comparable to the individuals harboring LGD variants, individual 36, harboring a *de novo* p.Arg841Leu variant, had GDD, autistic features, food allergies, and asthma. Immunodeficiency, either SCID or ICL, was observed exclusively in individuals harboring a missense variant affecting the “specificity residue” at amino acid position +3 (p.Asn441Lys, p.Lys469Asn, p.Asn810His, and p.Lys838Thr) (Figure 2 and Table 2). Similarly, brain MRI abnormalities, gait abnormalities, and seizures, rarely observed in individuals harboring an LGD variant, were more common (>50%) in individuals harboring a “specificity residue”-affecting missense variant. Dystonia, involuntary movements, and cerebral palsy were observed in both individuals harboring the p.Lys838Arg variant, which was extremely rare in individuals harboring an LGD variant (3%–6%). These data suggest that such missense variants exert a more severe effect than the LGD variants on the protein function. Interestingly, the reporter assay revealed that whereas all of the analyzed missense variants affecting a “specificity residue” impair DNA-binding affinity in a similar manner, they

differ in seemingly target-specific dominant-negative effects. The clinical variability is likely further explained by alterations in DNA-binding specificity as revealed by the modeling approaches (Figures 4 and S3) and already confirmed for p.Asn441Lys.<sup>30</sup> The latter is further supported by the clinical phenotype of individuals harboring a missense variant at residue Lys838. Namely, individual 33, harboring p.Lys838Thr, did not develop signs of dystonia, involuntary movements, and cerebral palsy, which were observed in both individuals harboring p.Lys838Arg. Similarly, in comparison to individual 33, both individuals harboring p.Lys838Arg did not develop SCID.

Taken together, the clinical, genetic, molecular, and modeling data suggest that the phenotypic diversity of *BCL11B*-related disorders is strongly dependent on the impact of diverse *BCL11B* alterations on its DNA-binding affinity and specificity. LGD variants, resulting either in haploinsufficiency or truncated proteins, with loss of at least the last two C-terminal C2H2-ZnFs leading to reduced DNA-binding affinity, cause a mild to moderate neurodevelopmental delay with increased propensity for behavioral and dental anomalies, allergies, and asthma, and reduced type 2 innate lymphoid cells. A similar clinical course is observed in individuals harboring a missense variant affecting zinc-binding residues, cysteines, and histidines within C2H2-ZnFs. Such missense variants result in reduced DNA-binding affinity and may additionally exert a dominant-negative effect in a target-dependent manner. However, missense variants affecting the DNA-contacting  $\alpha$  helix within a C2H2-ZnF that affect neither a zinc-binding residue nor a “specificity residue” result in variable clinical outcomes either similar to those of LGD variants or much milder and later in onset. The comparison of clinical data and results obtained by luciferase assay for other *BCL11B* alterations strongly suggest that their clinical outcome mostly depends on their DNA-binding affinity. The largest clinical heterogeneity and most severe clinical outcomes are observed in individuals harboring a missense variant affecting one of the four “specificity residues” within C2H2-ZnFs. Such missense variants result in reduced DNA-binding affinity and exert a dominant-negative effect in a target-dependent manner. In addition, however, missense variants affecting “specificity residues” result in binding to alternative DNA targets, thus resulting in altered expression of various genes that are not governed by WT *BCL11B*. Currently, it is impossible to predict such genes and how they will be altered due to such missense variants. Therefore, especially in a prenatal setting, it is impossible to predict their clinical outcome.

In addition to the three suggested *BCL11B* subtypes, an individual affected by syndromic GDD with craniosynostosis harboring a *de novo* p.Arg3Ser was recently described.<sup>34</sup> The identified missense variant, located within a conserved amino terminus, leads to impaired interaction with the RBBP4-MTA1 complex. It is conceivable that missense variants affecting further specific functional sites within *BCL11B*, e.g., affecting phosphorylation

sites or protein-interacting motifs, might further expand the clinical spectrum.

A further emerging question regarding individuals harboring a causative *BCL11B* variant remains the potentially increased risk for developing a malignancy. The analysis of murine models with *Bcl11b* alterations<sup>17,25</sup> and the somatic data of hematologic malignancies<sup>26–29</sup> suggest that *BCL11B* may act both as a tumor suppressor and as an oncogene. We report here two individuals harboring *BCL11B* variants, a frameshift variant and p.Ser836Asn, who developed a hematologic malignancy. However, it is arguable whether an incidence of ~2% (2/92) allows establishment of *BCL11B* as a tumor predisposition gene. The fact that both developed a different type of malignancy, namely T cell large granular lymphocytic leukemia and T cell acute lymphoblastic leukemia, does not currently allow recommendation of meaningful surveillance strategies.

Beyond the interfamilial variability in individuals 27–30, harboring p.Cys808Arg, several identical *de novo* variants result in variable clinical presentation. All three individuals harboring heterozygous p.Gly630Thrfs\*91 included in this study developed a skull abnormality. However, they are very different. Individual 9 had scaphocephaly, individual 10 developed secondary microcephaly, and individual 11 had macrocephaly. Moreover, an individual harboring p.Asn441Lys identical to the initially described *BCL11B* variant<sup>30</sup> had a somewhat milder clinical course, especially in terms of immunodeficiency, compared to the initial case.<sup>32</sup> Similarly, despite harboring the identical p.Asn807Lys variant, neither the here described individual 26 nor the previously reported individual<sup>38</sup> developed a congenital erosive dermatitis that we have documented previously.<sup>31</sup> Likewise, despite many similarities between individuals 23 and 24, both harboring *de novo* p.Lys469Asn, only individual 23 developed pigmentary mosaicism of the skin and white hair, which may be explained by the different nucleotide substitution c.1407G>T and c.1407G>C. Obviously, we cannot completely exclude the possibility that some of these individuals harbor additional variants or epigenetic alterations that may have modified the phenotype. It is worth noting, however, that DNA-binding affinity and specificity of transcription factors can additionally be altered due to changes within the DNA-binding regions, even resulting in cell-type-specific binding changes.<sup>63</sup> Such alterations, in particular single nucleotide polymorphisms, are thought to underlie the increased risk for common diseases.<sup>64</sup> It is thus reasonable to hypothesize that at least some of the observed clinical variability between individuals harboring an identical *BCL11B* variant might be due to rare or even common alterations within its target sites. Since *BCL11B* regulates gene expression in a context-dependent manner,<sup>9,10</sup> several integrated multi-omics approaches, including ChIP-seq, whole-genome sequencing, methylation profiling, and RNA sequencing, preferentially performed in various cell types and cell lineages, are required

to further elucidate the molecular mechanisms underlying the *BCL11B* phenotypic spectrum. Clearly, such analyses will require extensive further work and will be the main aim of our future studies.

In summary, by comparing the clinical and genetic data of altogether 92 affected individuals harboring a likely pathogenic or pathogenic *BCL11B* variant, we have expanded the known clinical spectrum. Combination of immune phenotyping, ChIP-seq followed by luciferase assays, and molecular modeling suggest that the broad phenotypic spectrum of *BCL11B*-related disorders is mainly due to the differential impact of the variants on its DNA-binding affinity and specificity. Our data further highlight the possibility that specific *BCL11B* missense variants can result in unpredictable clinical outcomes differentially affecting the immune system along with development and function of the brain, skin, and teeth.

## Consortia

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### Data and code availability

This article includes all datasets generated or analyzed during this study. The raw next-generation sequencing data that support the findings in affected individuals cannot be made publicly available for reasons of patient confidentiality. Qualified researchers may apply for access to these data, pending institutional review board approval (contact D.L., [davor.lesell@klinik.uni-regensburg.de](mailto:davor.lesell@klinik.uni-regensburg.de)).

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### Author contributions

I.L. and D.L. generated and analyzed the functional data. A. Baresic analyzed the ChIP-sequencing data. J.M. and E.T. generated and analyzed the immune phenotype data. N.M. and D.S. performed cell sorting of frameshift variants. I.L., A. Baresic, E.T., and D.L. wrote the manuscript. The remaining authors provided primary biomaterial (analyzed by J.M. and E.T.) and clinical and genetic data (analyzed by I.L. and D.L.). All authors discussed results, commented on the manuscript, and approved the final manuscript.

### Declaration of interests

Baylor College of Medicine (BCM) and Miraca Holdings Inc. have formed a joint venture with shared ownership and governance of the Baylor Genetics (BG), which performs clinical exome sequencing. J.R.L. has stock ownership in 23andMe, is a paid consultant for Genome International, and is a co-inventor on multiple United States and European patents related to molecular diagnostics for inherited neuropathies, eye diseases, genomic disorders, and bacterial genomic fingerprinting. A. Begtrup is an employee of and may hold stock in GeneDx, LLC.

### Supplemental information

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## Supplemental information

### DNA-binding affinity and specificity determine

### the phenotypic diversity in *BCL11B*-related disorders

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## Supplemental information

### Supplemental Note: Case Reports

#### Individual 1

This is a 12-year-old male, born at term by cesarean section to unaffected, non-consanguineous parents after an uneventful pregnancy. Family history is unremarkable. He had a birth weight of 3910 g (+0.7 SD), a birth length of 54.6 cm (+0.9 SD) and an Occipital Frontal Circumference (OFC) of 36 cm (+0.3 SD). The early postnatal period was characterized by feeding difficulties due to latching problems and poor sucking. He transitioned to solid foods without concern at 14 months of age. Growth parameters and early developmental milestones were unremarkable except for tongue hypotonia and speech impairment, which required speech therapy. Asthma was diagnosed at 12 months of age without any evident triggers, and attacks occurred randomly and suddenly. He required a nebulizer and due to asthma exacerbations underwent emergency interventions on several occasions. The situation gradually improved and he had no asthma attacks after 5<sup>th</sup> year of life. At the age of 4 years he was diagnosed with congenital unilateral left renal agenesis subsequent to frequent urinary tract infections, and also had multiple right kidney cysts. Dysmorphic facial features included long myopathic face, thin eyebrows, mild hypertelorism with heavy upper eyelids, elongated nose with hypoplastic alae nasi, long, flat philtrum, high narrow palate, and flat mid-face. Dental defects included absent permanent upper and lower lateral teeth and upper second premolars. At his last clinical examination at the age of 12 years his height was 153.7 cm (+0.1 SD) and weight 40.2 kg (-0.2 SD, BMI 16.6kg/m<sup>2</sup>). He was in good health except for slowly declining kidney function and borderline hypertension. He had several behavioral issues including stuttering, attention deficit hyperactivity disorder (ADHD), anxiety and impulsivity. He struggles with memory retention, requiring assistance for math and reading. He has trouble with fine motor tasks such as writing. He is sociable, and active in sports and able to perform all activities of daily living. A solitary cystic kidney, left renal agenesis was detected on MRI. Chromosomal analysis revealed an unremarkable male karyotype and array CGH did not identify any pathogenic copy number variants (CNV). Trio whole exome sequencing (trio-WES) with DNA samples of both unaffected parents and the proband, revealed a *de novo* heterozygous 7-bp deletion, c.183\_189delTCAAATG, in *BCL11B* (NM\_138576.3) resulting in direct premature stop codon p.(Cys61\*).

#### Individual 2

This is a 13-year-old female, the second/third child of unaffected non-consanguineous parents (from Georgia). She was born at full term by vaginal delivery after an uncomplicated pregnancy. Infancy was characterized by thrombocytopenia and neutropenia from 8 months of age. Bacterial infections were frequent during the first years of life (probably related with neutropenia). At the age of 7 and 8 years, she had several infections by Herpesviridae (EBV, VZV, HS). She had evidence of unremarkable serum immunoglobulin levels including protective responses to tetanus vaccine, pneumococcal conjugate vaccine, measles vaccine and COVID Vaccine. She also had evidence of IgG to Epstein-Barr virus. She sat at 8 months, and started to walk at 15 months. She spoke first words at 10 months; however she spoke only short sentences at 4 years of age (with logopedics help). She is attending a school, and is able to read and write, and was diagnosed with an autism spectrum disorder. Physical exam showed

hypertelorism, low set ears and short philtrum. She was reported to have eosinophilia, but neither asthma nor allergies. MRI of the brain showed no pathological characteristics. Trio-whole exome sequencing (trio-WES) with DNA samples of both unaffected parents and the proband, revealed a *de novo* heterozygous 7-bp deletion, c.183\_189delTCAAATG, in *BCL11B* (NM\_138576.3) resulting in direct premature stop codon p.(Cys61\*).

### Individual 3

This is a 2 years 5 months old female, born at term after an uneventful pregnancy with unremarkable body measurements to unaffected non-consanguineous parents. She has two unaffected siblings. At birth a head hematoma, reduced muscle tone and pes planovalgus were observed. EEG and cMRI investigations revealed no abnormalities. Her development was markedly delayed. She started crawling at the age of 13 months, could barely stand with support but could not walk freely at 2 years and 5 months of age. She could say some 5 understandable words but had comparatively better language comprehension. She demonstrated stereotypic flapping hand movements. She has a regular sleeping pattern and no feeding problems. An ophthalmological assessment revealed hyperopia and strabismus convergens. Repeated EEGs and a second cMRI revealed no abnormalities. A number of dysmorphic features were apparent: a narrow brow, deep-set eyes, a broad nose, a long philtrum and an open mouth. SNP array showed no relevant copy number variants. Trio-WES with DNA samples of both unaffected parents revealed a *de novo* heterozygous 26-bp deletion, c.1460\_1485delGCTCCGACGACGGGCTCTCGGCCGCC, in *BCL11B* (NM\_138576.3) resulting in a frameshift variant p.(Arg487Glnfs\*21).

### Individuals 4 and 5

Individual 4 is 36-year-old affected female, mother of a male, 9 years and 10 months old similarly affected individual 5. Individual 4 reports that her developmental milestones were unremarkable. She graduated high school and had learning disabilities that required special education, but not to the extent of her son. Her medical concerns include irritable bowel syndrome, alopecia totalis, hypothyroidism, reflux, anxiety, and obsessive-compulsive disorder (OCD). She is missing upper teeth, has several allergies and asthma, and history of recurrent acute otitis media. Her son, individual 5, has a history of developmental delays, craniosynostosis, autistic features, and dysmorphic features including lateral 1/3 missing and hardly present eyebrows on the left, depressed nasal bridge, flat philtrum and dental crowding. She spoke first words at the age of 24 months and walked without support at 18 months of age. Mother reports on feeding issues and constipation. She has exotropia, exophthalmos and hyperopia. Immunologic analysis revealed a mild eosinophilia (5, ref. 0-3), slightly elevated level of T-Cells (79%, ref. 68-74%) and decreased level of B-Cells (10%, ref. 13-19). Array CGH of the individual 3 was unremarkable and WES analyses revealed a heterozygous 1-bp deletion, c.1474delC, in *BCL11B* (NM\_138576.3) resulting in a frameshift variant p.(Leu492Serfs\*71). Subsequent analysis revealed that this variant was inherited from the mother, individual 4, who did not inherit it from her parents.

### Individual 6

This is a 13-year-old male, the second of five children of unaffected, non-consanguineous parents. The family history is uneventful. The pregnancy was complicated by an increased risk of trisomy 21. He was born at term

with a birth weight of 3580g (+0.62 SD), birth length of 52.5 cm (+0.62 SD) and an OFC of 35 cm (+0.79 SD). Milestones of motor development were moderately delayed; she was walking at 18 months. Speech development was severely impaired, with only a few words spoken at the age of 3 years, and only short sentence at 4 years. She showed behavioral difficulties with tantrum which improved over time. She suffered from recurrent, mostly upper airway infections and asthma. She showed hypodontia and suspected enamel defects. At last clinical examination at the age of 13 years and 2 months his height was 157.5 cm (+1 SD), weight 35 kg (-1 SD), and OFC 51.5 cm (-2 SD). Facial dysmorphisms consisted of hypertelorism, upslanting palpebral fissures, hypoplastic nostrils, thin eyebrows, low hanging columella, long philtrum, and thin upper and lower lip vermillion. She has been in special education throughout the schooling. Brain MRI gave unremarkable results, Urinary organic acids, plasma amino acid chromatography and Fragile X studies were unremarkable. Microarray analysis revealed a 1.3Mb deletion at 17p12 (with PMP22), which was inherited from his mother. Trio-WES with DNA samples of both unaffected parents and the proband, revealed a de novo heterozygous 1-bp deletion, c.1552delC, in *BCL11B* (NM\_138576.3) resulting in a frameshift variant p.(Arg518Alafs\*45).

### **Individual 7**

This is a 5-year-old female, born at term to unaffected, non-consanguineous parents at 41 weeks of gestation after an uneventful pregnancy. Birth length, weight and OFC were in the mid- unremarkable range. The newborn period was uneventful. Global developmental delay was noticed during the second year of life. She learned to walk at 18 months with physiotherapy. At 5 years of age, she spoke two word combinations. Her body measurements at 5 years of age were height of 105.9 cm (-1,5 SD), weight of 16 kg (-1,5 SD) and OFC 48.5 (-1,9 SD). Later on she developed microcephaly. She had several behavioral issues including ADHD and concentration problems. Physical examination showed hypertelorism, long philtrum, prominent nose and dental caries. She had two episodes of generalized exanthema of unknown origin, last at the age of 5 years. Brain MRI revealed a thin corpus callosum and pituitary stalk lesions. EEG showed irregular amplitudes and frequencies. Cerebrospinal fluid analysis revealed a lymphocytic pleocytosis. Immunologic analysis revealed a mild eosinophilia and elevated levels of serum interleukin 2 receptor (IL2R). Skeletal age determination performed according to Greulich-Pyle method revealed a retardation of 4 years, and blood workup showed decreased growth hormone levels. She has strabismus divergens intermittens. At last clinical examination at the age of 13 years and 9 months her height was 132.5 cm (-4.3 SD), weight 26.9 kg (-4.5 SD), and microcephalic OFC of 50 cm (-3.8 SD). She was reported to have recurrent nighttime nausea and vomiting. Karyotyping and Array CGH showed unremarkable findings. WES of the proband revealed a heterozygous 1-bp deletion, c.1742delG, in *BCL11B* (NM\_138576.3) resulting in a frameshift variant p.(Gly581AlafsTer24). Sanger sequencing did not identify this variant in parental DNA samples and confirmed the heterozygous variant in the proband.

### **Individual 8**

This is a 9-year-old female, born at term to unaffected, non-consanguineous German parents at 39 weeks of gestation after an uneventful pregnancy. Birth length (50 cm; 31st centile), weight (3380 g; 54th centile) and OFC (34 cm; 32nd centile) were in the mid-unremarkable range. The newborn period was uneventful. Global developmental delay was noted at the age of 6 months. She learned to walk at 18 months with physiotherapy. At 3 years of age she spoke two word combinations. Since infancy, she is difficult to feed and only likes to eat specific food (high calory drinks, bread and other bakery products). At the age of three years, she experienced two systemic allergic reactions (including skin, intestine and throat) after the oral intake of banana and after the intake of pistachio. Later an allergy test revealed sensibilization to banana, chicken egg, soy and dog hair. She had overall developmental delay. Her development was delayed approximately by 2-3 years. She had overall gross and fine motor deficits. At the age of 9 years, she had learned to swim, she danced ballet, she learned to ride a scooter, but had problems with starting and stopping. At the age of 8 years, she was diagnosed with autism spectrum disorder. When exposed to new situations, she often stopped to speak and started to cry. She had repetitive and stereotypic behavior (for example collecting special gadgets with the same colors and talked to herself when playing). At the last examination, at the age of 9 years, she attended a regular elementary school and only needed specific support when for example writing math examinations. A change to an integrative school was planned. Her body measurements at 6 years of age were height of 110 cm (-1.84 SD), weight of 14,5 kg (-3.2 SD) and OFC 51.5 (mean). Physical examination showed a hypotonic facial appearance, hypertelorism, thin eyebrows, narrow palpebral fissures, long philtrum, prominent nose and thin upper lip. She had a few missing mispositioned and teeth and the enamel had a reduced quality (the dentists suspected caries). Immunologic analysis revealed unremarkable peripheral blood count. Brain MRI was not performed. Trio-WES with DNA samples of both unaffected parents revealed a 2-bp deletion, c.1770\_1771del; in *BCL11B* (NM\_138576.3) resulting in a frameshift variant p.(Lys591Glyfs\*293).

### Individual 9

This is a 7-and-a-half-year-old female, the first child of unaffected, non-consanguineous Caucasian parents. She was born in the 38th week of gestation, via cesarean section. The father was said to be a late-developing child, he began to stand at 20 months and walk at 22 months, but his performance improved in the adulthood. The pregnancy was complicated with fetal bradycardia noted from the 36th week. Birth weight was 3000g (-0.4 SD), APGAR was 9/10, birth length and OFC were not registered. Feeding was problematic since birth: swallowing difficulty with frequent choking was noted and several different formulas were tried to achieve comfort because of irritability, giving the impression of colic pain. Based on noisy breathing, laryngomalacia was suspected but no bronchoscopy was performed to confirm this. Hypotonia and mild developmental delay were seen in early infancy, special physiotherapy was initiated. She began to roll over at 5 months, unassisted sitting was achieved at 11 months, standing at 15.5 months, unaided walking at 20 months. Spasticity of the lower limbs were observed. Occasionally there was dystonic, volar-flected posture of the left hand causing difficulty to grab an object. The first meaningful word („mama”) was heard at 1 year of age; intelligible speech began at 4 years. From 4.5 years the child has been living in a bilingual environment. Convergent strabism was present from birth, severe visual impairment affects one eye. Ophthalmological therapy consisted of alternate covering of the eyes and surgical correction. The improved visual perception after the eye-surgery resulted in a better rate of general development, at 16 months developmental quotient according to the Bayley scale was 73, and 89 according to the Brunet-Lazine

scale with more delay in the gross and fine motor skills and less in the cognitive functions. At 17 months of age, two consecutive febrile infections occurred and lead to the worsening of the dysphagia. Fatigue also made the swallowing difficulty worse and it provoked ptosis and worsening of the strabismus, based on which myasthenia gravis was considered. Neurological examination at 18 months of age described dyscrania without craniosynostosis, strabismus spurius but otherwise unremarkable oculomotor functions, unremarkable swallowing, unremarkable muscle tone and strength, stereotypic hand movements. Between 4 and 5 years of age there were a few short seizures with oral automatisms consistent with Rolandic epilepsy which did not require therapy and hasn't recurred in 2 years. Brain MRI revealed scaphocephal skull shape, an „inka” bone, megacysterna magna, mildly dilated supratentorial CSF space and unremarkable myelination. It was repeated at 5.5 years and revealed no pathologies. At 18 months of age she had a weight of 10kg (-0.8 SD), length of 86cm (+1.2 SD) and OFC of 48cm (+0.5 SD). At 7.5 years parents reported overall good development with only mild developmental delay (IQ 65-70), the child is able to take part in integrated education in a mainstream school, with good bilingual verbal performance regarding both grammar and vocabulary skills, although there is occasional stammering. Oral motor function improved a lot but is still weak and causes articulation problems. Reflux esophagitis requires intermittent proton-pump inhibitor therapy. Eating and swallowing improved and became almost unremarkable. No behavioral issues are reported, the child has an overall friendly, cheerful disposition. Morphologically, the skull is bitemporally narrow, there are upslanted and vertically narrow palpebral fissures, low nasal root, broad nasal base, long philtrum, widely spaced teeth, and rounded nails on the fingers. Early enamel decay necessitated the removal of several deciduous teeth. Testing for nutritional allergies are pending because of intermittent urticarias. Array CGH gave an unremarkable female karyotype. WES of the proband revealed a heterozygous 7-bp deletion, c.1887\_1983del CCGCGGG, in *BCL11B* (NM\_138576.3) resulting in a frameshift variant p.(Gly630Thrfs\*91).

### Individual 10

This is a 6-year-old female, the only child of unaffected, non-consanguineous parents. Family history is unremarkable. She was born at 41+5 weeks, following an uneventful pregnancy. Birth weight was 3020 g (-0.97SD), length 48 cm (-1.37SD), cc 33 cm (-1.16SD). APGAR was 9-10. Besides feeding difficulties due to poor sucking, the perinatal period was uneventful. In terms of development, she was able to sit without support at 8 months and walk at 18 months. She was toilet trained by the age of 3 and was able to spell 6 words at the age of 4 years. However, according to the last follow-up, parents report language regression and loss of bladder and bowel control. Since early childhood she has several behavioral issues, including anxiety, auto- and hetero-aggressive behavior, bruxism's and sleeping issues. She experienced four upper respiratory infections during the last year. White blood count and immunoglobulin level were unremarkable. Moreover, parents report multiple dental caries and constipation in part due to a selective diet. Feeding difficulties improved over years. At the last evaluation at the age of 6 years, she had a height of 110 cm (-1.7 SD), weight of 14 kg (-2.8 SD) and OFC of 47 cm (-3.1 SD). The dysmorphological assessment revealed short forehead, thin eyebrows, “beaked nose” with narrow nasal tip, bilateral earlobe hypoplasia, small mouth with lips, horizontal crease of chin, clinodactyly of II toe and V finger bilaterally, hypertrichosis of the back. Several investigation including brain MRI and metabolic screening gave unremarkable results. Array-CGH and a NGS panel (including 33 genes) associated to short stature yielded negative results. Similarly, sequencing and MLPA analysis of EP300 and CREBBP for the suspicion of Rubinstein-Taybi were unremarkable. Trio-WES with DNA samples of both unaffected parents and the proband,

revealed a *de novo* heterozygous 7-bp deletion, c.1887\_1893delCGGCGGG, in *BCL11B* (NM\_138576.3) resulting in a frameshift variant p.(Gly630Thrfs\*91).

### Individual 11

This 24-year-old woman is the second child of unaffected, consanguineous parents. She was born after an uncomplicated pregnancy and unremarkable amniocentesis for maternal age. Mom was induced at 42 weeks and her birth weight was 3941g (+0.7 SD). She had a history of feeding difficulties, failure to thrive and hypotonia. She walked at 23 months and had three words at 2 years 5 months. As a child, she was assessed by Genetics and Pediatrics for developmental delay, intellectual disability, recurrent infections and facial dysmorphisms. Her workup included unremarkable metabolic testing, karyotype and microarray, screening for Smith-Lemli-Opitz syndrome, and negative testing for Fragile X, Angelman syndrome, Williams syndrome and X inactivation studies. No diagnosis was made. She had ongoing speech, occupational and physiotherapy as well as educational assistance in school. She was described as a happy and social child. At age 12, she developed Graves disease and was found to have mitral valve prolapse and developed dystonia in her teens. Her hands and feet hypertonia cause her pain for which she received orthotics help. Physical exam as a child showed delayed tooth eruption, dental crowding, bilateral coxa valga. Her distal phalanges were short with radial deviation brachydactyly and early fusion of epiphyses. The last physical examination at age 21, revealed hypertelorism as well as short, down-slanting palpebral fissures, thin eyebrows, and a broad nasal bridge. She had a long philtrum, thin upper lip and small mouth. Her head circumference was over average for a female at 59.5cm (+3SD). She had oligodontia and small teeth; clinodactyly and hyperextensible hand joints were also identified. Her dystonia worsened during her physical exam. She has scoliosis and pes cavus. She attended a day program four days a week and volunteered. She was dependent on her parents for daily living activities such as finances and preparing meals. She had multiple allergies. Her neurological exam revealed stereotypies of her upper limbs and dystonia of her lower extremity as well as swan neck deformity of some of the distal interphalangeal joints in the hands. Abduction of the toes was observed as well as abnormal posturing and in-turning of the right foot. Trio-WES with DNA samples of both unaffected parents and the proband, revealed a *de novo* heterozygous 7-bp deletion, c.1887\_1893delCGGCGGG, in *BCL11B* (NM\_138576.3) resulting in a frameshift variant p.(Gly630Thrfs\*91).

### Individual 12

This is a 6-year-old female, born after an uneventful pregnancy via vaginal delivery with a birth weight of 2807g. The proband's parents are non-consanguineous. Her father and her three paternal half-siblings have non-syndromic mild to moderate intellectual disability. In the first week of life she was on Neonatal Intensive Care Unit because of feeding difficulties and gastro-esophageal reflux. She achieved independent sitting at 6 months, cruised at 13 months and walked at 19 months of age. She started babbling under the age of a year and by age 18 months had a number of single words. She has autistic traits and poor sleep pattern. She receives one-to one support and attends a Special Needs Unit within a mainstream school. Ophthalmology assessment identified hypermetropic astigmatism. She has not suffered severe, unusual, recurrent or persistent infections, and there was no history of thrush, warts or cold sores. She had chicken pox at 5 years which followed a typical course. She required tympanostomy tube insertion aged 4 years for otitis media with effusion. She has mild atopic eczema and has suffered nut allergy with facial swelling and urticaria. Physical examination showed small up slanting palpebral

fissures, thin eyebrows, a high anterior hairline, a broad forehead, a short nose with anteverted nostrils, a long philtrum and small mouth with thin lips and down turned corners. Her ears were pointed in shape, posteriorly rotated and low set. She had a somewhat short neck with low posterior hairline. Her hands and feet appeared small with mild brachydactyly and fifth finger clinodactyly and bilateral single palmar creases. Analysis of her peripheral blood revealed an unremarkable immunophenotype. This included unremarkable enumeration of T cells (including naïve T cells) and T cell receptor excision circles, as well as B cells (including B cell maturation marker expression). She had evidence of unremarkable serum immunoglobulin levels including protective responses to tetanus vaccine, pneumococcal conjugate vaccine, Haemophilus influenzae type b vaccine and measles vaccine. She also had evidence of IgG to Epstein-Barr virus without an accompanying history of this infection. At 20 months, her head circumference was 45.6cm (-2.8), weight was 9.08kg (-2.1 SD) and her height was 72 cm (-3.9). At 8 years of age, her weight was 26.4kg (-0.2 SD), height was 116.5cm (-2.4), head circumference was 52.4cm (-0.4 SD). Trio-WES with DNA samples of both unaffected parents and the proband, revealed a *de novo* heterozygous 22-bp deletion, c.1944\_1965delCGGCGCGGTCAACGGGCGCGGG, in *BCL11B* (NM\_138576.3) resulting in a frameshift variant p.(Gly649Alafs\*67).

### Individual 13

This is a 15-year-old female, the first child of unaffected, non-consanguineous parents. She was born after an uneventful pregnancy at term with a birth weight of 3855 g (+1.0 SD). She had global developmental delay and was diagnosed with asthma in early childhood. She walked at 18 months of age and was able to speak in sentences in Reception year at school. She was reported to have many respiratory infections in childhood. She was diagnosed with a refractive error in Primary school. Significant regression with gradual changes in her mood and behavior (low mood accompanied by social withdrawal and reduced motivation) as well as somatic (sleep disturbance, loss of appetite and double incontinence) were noted at 10 years of age. Her behavior gradually worsened as she developed repeated episodes of self-harm, smearing and eating non-edible objects such as feces. From around 13 years of age she developed episodic changes in mood and behavior with an apparent regular cycle. Each episode may last 2-3 weeks and include: a “low mood” episode, an “aggressive” episode and a “happy” episode. Growth parameters at 14 years of age: weight 53kg (+0.35 SD), height 161cm (+0.21 SD). Currently at age 15 years she is considered to have moderate degree of learning disability. For communication in school she uses mainly symbols whereas at home she can use short phrases. She is fully ambulant but uses a wheelchair for longer distances. Her CGH-array, brain MRI scan and metabolic investigations were unremarkable. Facial dysmorphisms included sparse lateral aspects of eyebrows, hypertelorism, short palpebral fissures, prominent nose, and thin upper lip vermilion (for her ethnic background). Trio-WES with DNA samples of both unaffected parents and the proband, revealed a *de novo* heterozygous 1-bp deletion, c.1988delA, in *BCL11B* (NM\_138576.3) resulting in a frameshift variant p.(Glu663Glyfs\*60).

### Individual 14

This is a 19-year-old male, the first child of unaffected, non-consanguineous parents (mother was 20 and father 28 years at the birth). He was born at full term with a birth weight of 2750 g (-1.1 SD). He has two unaffected siblings, and his mother required speech therapy for articulation. A sagittal and bilateral lambdoid craniosynostosis was

observed at birth. His early development was characterized by developmental delays. He learned to walk at the age of 2 years and in non-verbal at the age of 19 years. Facial dysmorphism include brachycephaly, upwardly slanted palpebral fissures, epicanthal folds, broad nasal bridge, long nose, downturned nasal tip, short columella, long philtrum and thin upper vermillion. Engages in repetitive behaviors (plays with beads on strings), occasional self mutilative behavior, easily angered. Due to sensory apraxia only drinks liquids. He has a hyperpigmentation. CT of head was unremarkable. Currently being seen by Hematology/Oncology for T-cell large granular lymphocytic leukemia and pancytopenia whose extensive workup for etiology has been negative to date. At last examination he had a weight of 56.9 kg (-1.7) and a height of 166.9 cm (-2.01 SD). Genetic workup prior to WES revealed an unremarkable karyotype and a 17p triplication. Singleton-WES revealed a VUS in *CACNA1G*: c.2479G>C, p.Gly827Arg VUS and a pathogenic variant in *BCL11B*: c.2119\_2126delGTGTACTCinsA, resulting in a frameshift variant p.(Val707Serfs\*14). Sanger sequencing did not identify this variant in parental DNA samples and confirmed the heterozygous variant in the proband.

### Individual 15

This is a 12-year-old female, the second child of unaffected, non-consanguineous parents. After an uneventful pregnancy, she was born at term, with a birth weight of 3310 g (-0.4 SD), a birth length of 50 cm (+0.0 SD) and an OFC of 34 cm (-0.5 SD). The family history is uneventful. She had an unremarkable neonatal period. She was able to walk at 16months. Speech development was impaired, with only a few words spoken at age 2 years and 6 months. At her last clinical examination at the age of 12 years and 10 months her height was 157 cm (+0.5 SD), weight 61.4 kg (+0.3 SD), and OFC 54.5 cm (+1.0 SD). Facial dysmorphism include a long nose with protruding columella, hypoplastic alae nasi, thin upper lip vermillion, low set ears. She had short hands, bilateral V clinodactyly and mild hypertrichosis. The parents reported severe behavior problems resembling autism, aggressivity, hyperphagy and severe sleep problems requiring medication. MRI of brain and hearing testing were unremarkable. Learning difficulties requiring special education. She was also followed for food allergies. Chromosomal analysis and an array-CGH analysis gave an unremarkable female karyotype, *FMRI* analysis gave unremarkable results. Trio-WES with DNA samples of both unaffected parents and the proband, revealed a *de novo* heterozygous 1-bp deletion, c.2348delG, in *BCL11B* (NM\_138576.3) resulting in a frameshift variant p.(Gly783Alafs\*2)

### Individual 16

This is a 45-year-old male, the second of four children of unaffected, non-consanguineous parents. He was born at term with a birth weight of 3150g (-1.0 SD) and a birth of height 49.5cm (-1.3 SD). The early postnatal period was characterized by muscular hypotonia. At one year of age hydrocephalus was suspected. He learned to walk at 1.5 years of age. He had a couple of words. At three years of age craniosynostosis (sagittal suture) was operated. At 7 years of age his developmental level was estimated to be at 3 years. Strabismus has been operated at 7 years of age. Currently at the age of 45 years he has moderate ID with no speech. His adult height is 157 cm (-3.5 SD), weight 66.5kg (-0.43 SD) and head circumference 54 cm (-1.99 SD). Epileptic fits started at the age of 26 years. Hypothyroidism was diagnosed as adult. He has encopresis and enuresis. He has severe fish allergy but no history of recurrent infections. His behavior is characterized by restlessness, anxiety and aggressive bursts. Clinical evaluation showed myopathic facies, low-set ears, prominent nose, long philtrum, thin upper lip, and his posture

is characterized by flexion of the trunk. Analysis of urine amino acids, as well as cytomegalo-, toxoplasma-, listeria- and rubeola -antibodies gave unremarkable results. Chromosomal analysis and an array-CGH analysis gave an unremarkable male karyotype. Trio-WES with DNA samples of both unaffected parents and the proband, revealed a *de novo* heterozygous 1-bp deletion, c.2448delG, in *BCL11B* (NM\_138576.3) resulting in a frameshift variant p.(Ser817Alafs\*27). In addition, a *de novo* 3-bp deletion, c.756\_758delAGA, in *CSNK2A1* (NM\_177559.3) resulting in in-frame deletion p.(Glu252del) was identified. The latter was classified as “variant of unknown significance” according to the American College of Medical Genetics (ACMG) guidelines.

### Individual 17

This is a 20-year-old male who first presented for evaluation in a genetics department at 13 years and 7 months due to an abnormal brain MRI, history of gastroesophageal reflux disease (GERD), and developmental delays. His prenatal and birth history are remarkable for premature delivery at 32 weeks gestational age secondary to maternal HELLP syndrome. He was 1701g (-0.7 SD) at birth and was a dizygotic twin pregnancy. He spent the first 2.5 weeks in the neonatal nursery. He underwent Nissen fundoplication at 2 years of age due to GERD, which had to be repeated at 5 years of age due to a large paraesophageal hernia and reflux. His formal IQ testing measured IQ of 60. Brain MRI revealed a foreshortened slab-like appearing corpus callosum with cerebellar ectopia and a borderline Chiari I malformation. He also has a history of eczema, alopecia areata, bilateral amblyopia, vitamin B12 deficiency, folate deficiency, anxiety and self-injurious behaviors with several attempts at self-harm. He has difficulties with sleep and more recently had concerns with cognitive regression. He has numerous food allergies. Physical exam findings include a double posterior hair whorl, dolichocephaly, sparse eyebrows, prominence of the supraorbital regions, frontal bossing, hypertelorism, mild ptosis bilaterally, borderline low set ears with simple architecture, high arched palate, retromicrognathia, mild pectus excavatum with carinatum at the top, increased carrying angle, and 2,3 toe syndactyly. Immunologic analysis revealed a mild eosinophilia with 7 per 10<sup>6</sup>/mL (ref. Range <6), elevated total serum immunoglobulin E (IgE) of >2000kU/L (ref. range <88) and slightly low B cell count of 99cells/ $\mu$ L (ref. range 107 - 698). Trio-WES with DNA samples of both unaffected parents and the proband, revealed a *de novo* heterozygous 14-bp deletion, c.2448\_2461delGAGCCACACCGGCG, in *BCL11B* (NM\_138576.3) resulting in a frameshift variant p.(Ser817Alafs\*63).

### Individual 18

This is a 3-year-old male, born at 33 weeks gestation to non-consanguineous parents. He is the product of a di-di twin pregnancy that was conceived using IVF with parent gametes. Ultrasounds during the pregnancy showed IUGR as compared to the twin. He weighed 1928 grams (-1.57 SD) and was 43.18 cm 8-1.71 SD) in length with an OFC of 33.5 cm (+0.25 SD). He required CPAP after birth due to prematurity and spent 25 days in the NICU. Muscle tone was noted to be unremarkable in the newborn period. He had bilateral inguinal hernias that required surgical repair. Subsequently noted to have hypotonia by neurology at 17 months of age. He had feeding difficulties as a newborn resulting in poor weight gain. Evaluation by ENT found a grade 1 laryngeal cleft, as well as issues with dysphagia and reflux resulting in aspiration with feeds, which improved with conservative measures. Motor milestones were globally delayed early on, though independent walking was achieved at 14 months, but with a notable crouched gait. Evaluation by orthopedics showed no hip dysplasia but confirmed hip flexion contractures and overall stiffness of the lower extremities, and a clinical diagnosis of arthrogryposis was given. MRI brain and

spine was done at 21 months. MRI spine was unremarkable, however the MRI brain showed faint bilateral T2 hyperintense signal changes of the central tegmental tracts. He is delayed in expressive language with apparently unremarkable receptive language. Genetics evaluation noted mild dysmorphisms including midface flattening, flattened nasal bridge with rounded nasal tip, micrognathia, very narrow and high palate with dental crowding and a small mouth with decreased opening range. He received a diagnosis of spastic diplegic cerebral palsy from orthopedics based on his contractures. He was evaluated by rheumatology due to joint pains. MRI pelvis and ankles showed mild synovial thickening throughout multiple joints of the midfoot and hindfoot without joint effusions. Also noted focal, subtle enhancement of the tendon sheath about the peroneus tendon just posterior to the lateral malleolus, suggestive of inflammatory arthropathy. At 24 months, his height was 83 cm (-1.43 SD), weight was 13.1 kg (+0.61 SD) and OFC was 50.5 cm (+1.64 SD). SNP microarray was unremarkable, and a comprehensive arthrogryposis panel was non-diagnostic. Trio WES was done which showed a de novo heterozygous 14 bp deletion, c.2439\_2452del in *BCL11B* (NM\_138576.3) resulting in a frameshift variant p.(Ser817Alafs\*63).

### Individual 19

This is a 10-and-a-half-year-old female, the first child of unaffected, nonconsanguineous parents. She was born after an uneventful pregnancy at 38 weeks of gestational age by cesarean section. Her birth weight was 2,615 g (+0.1 SDS), birth length 45 cm (-1.3 SDS), and OFC 33 cm (+0.2 SDS). It was observed in the first year of life a developmental delay, poor growth and severe food allergy. She was able to hold head at 7, sit without support at 12, and to walk and speak first word at the age of 18 months. She was diagnosed with milk and egg allergies in the first year of life. She had frequent upper respiratory infections and developed a moderate asthma. A primary autoimmune hypothyroidism was diagnosed at age of 5 and adequately treated. At age of 7.3 years her height was 108.5 cm (-2.8 SDS), weight was 16.3 kg (BMI SDS of -1.3), a head circumference of 50 cm (-0.8 SDS) and unremarkable body proportions. She started treatment with growth hormone to improve her height, with good response. At the last visit, at the age of 10.5, her height was 132 cm (-1 SDS), she is growing 8.4 cm/y and just started puberty. She has several dysmorphic features including low-set posteriorly rotated ears, small palpebral fissures, prominent nose with broad nasal tip, thin upper lip vermilion, high arched palate and pointed chin. She attends a regular school in inclusive education way, but has a major learning disability; she is not yet able to read, write or perform calculations. Immunologic analysis revealed an eosinophilia and elevated IgE levels (>5000KU/L). During the current COVID-19 pandemic, she got infected and had the disease in a mild form. WES with DNA of the proband revealed a heterozygous 14-bp duplication, c.2439\_2452dupGCACCGCGGAGCC, in *BCL11B* (NM\_138576.3) resulting in a frameshift variant p.(His818Argfs\*31). Sanger sequencing did not identify this variant in parental DNA samples and confirmed the heterozygous variant in the proband.

### Individual 20

This is a 7 year and 9 month old female, the fourth child of unaffected, nonconsanguineous parents. She was born via IVD to a 27-year-old G4P3Ab1 at 37 weeks gestation following a pregnancy complicated by late oligohydramnios and concern for decreased foetal movements. Her birth length was 53.34cm (+1.6 SD) and birth weight of 3374g (+0.9 SD), and she was discharged at 2 days of age with no immediate concerns. She first came to medical attention at the age of 2 years, when she was noted to have developmental delay: she walked independently and spoke first words at 27 months. Her gait was notable for right foot in-toeing. At 4 years, 11

months of age, her weight was 17.2 kg (-0.6 SD), her height was 105.5 cm (-1.0 SD) and had a head circumference of 49 cm (-1.3 SD). Physical exam was notable for telecanthus, shortened palpebral fissures, a thin upper lip vermilion, and a flat philtrum. Her most recent evaluation was at 7 years and 9 months, at which time she was noted to have dysmorphic craniofacial features as noted above, developmental delay, an in-toeing gait, and brief staring spells believed to be absence seizures. At that time, her family further reported severe constipation with once-weekly bowel movements, and recent diagnoses of amblyopia and strabismus, as well as eczema, allergic rhinitis and asthma. A SNP-array was unremarkable. Trio-WES with DNA samples of both unaffected parents and the proband, revealed a *de novo* heterozygous 14-bp duplication, c.2439\_2452dupGGCTCCGCCGGTGC, in *BCL11B* (NM\_138576.3) resulting in a frameshift variant p.(His818Argfs\*31).

### **Individuals 21 and 22 (siblings)**

#### **Individual 21**

This is a 9 year 3 month old girl, born to non-consanguineous parents of Ashkenazi Jewish descent. Mother was taking synthroid® during the pregnancy due to hypothyroidism. She was born full term. Birth measurements were unknown, and no complications were reported after birth. She had feeding difficulties in infancy and had difficulty latching. She also initially had difficulties gaining weight. She had allergies when younger that resolved over time. Developmental delays were noted at 9 months of age. She walked just before 2 years of age, and first words came around 4 - 4.5 years of age. At 9 years of age, she could put words together, but speech was not clear. She continued to receive therapies since infancy. She was able to read but writing was difficult. She also struggled socially. At 2 years old, she was diagnosed with verbal apraxia. She has never had any seizures and she had never had any neuroimaging studies. Hearing and vision screens were unremarkable. Most recent clinical evaluation at 9 years 3 months was significant for slight hypertelorism, narrow downslanted palpebral fissures, broad middle part of the nose, flat midface with high square forehead, elongated face, and upturned corners of the mouth. Height was 127 cm (-1.61 SD), weight was 24.9kg (-1.37 SD), and OFC was 51.5cm (-0.71 SD). Family history was significant for a similarly affected sister (individual 21).

#### **Individual 22**

This is a 2 year 5 month old girl, born to non-consanguineous parents of Ashkenazi Jewish descent. Mother was taking synthroid® during this pregnancy due to hypothyroidism. She was born full term. Birth measurements were unknown. The baby was initially taken to the NICU after birth for approximately 12 hours and then discharged home with the mother. The reason for the NICU admission was unknown. Developmental delays were first noted around 18 months of age. She started to walk when she was more than 2 years old. At 2 years 5 months old, she was babbling, but did not have any words yet. She was receiving multi-modal therapies. The parents believed that she could understand well. She was generally in good health. She had allergies to nuts, milk, and eggs. At most recent clinical evaluation at 2 years 5 months, clinical features were notable for tall square forehead, flat midface, hypertelorism, small upturned nose, and upturned corners of the mouth. Weight was 11kg (-1.22 SD), height was 81.6cm (-2.39 SD), and head circumference was 48.3cm (-0.49 SD). Family history was significant for a similarly affected sister (individual 20). She also had seven additional unaffected siblings. Quad exome sequencing at GeneDx with the similarly affected sister (individual 20) showed the two affected siblings to be heterozygous for a 1-bp deletion, c.2499delG, in *BCL11B* (NM\_138576.3) resulting in a frameshift variant p.(Cys833Trpfs\*11). Both parents tested negative via saliva samples, which strongly suggested mosaicism in one of them. Microarray

testing revealed a paternally inherited 386 kb duplication at 3p25.2 (12484848\_12871176) of unknown clinical significance. Her affected sister (individual 20) is also heterozygous for the 3p25.2 duplication.

### Individual 23

This is a male who was born by spontaneous vaginal delivery at 38+5 weeks gestation. Pregnancy was complicated by maternal coeliac disease and flu infections. Prenatal medications included Buspar, Zofran, prenatal vitamin, Zantac. First trimester screen and ultrasound imaging were unremarkable. His birth weight was 3030 g (-0.5 SD), birth length was 48 cm (-1.1 SD) and birth OFC was 32 cm (-2.0 SD). After delivery infant was noted to have tachypnea within hours of delivery and was admitted to the hospital NICU. During his 39-day NICU stay he was noted to have dysmorphic features, oral feeding problems and muscular hypotonia. MRI brain indicated agenesis of the corpus callosum. He had gastrostomy tube placed. After discharge home from the NICU he was admitted to the children's hospital at 3.5 months old due to respiratory distress with cyanosis concerning upper airway obstruction. He was found to have severe micrognathia with submucous cleft palate with abnormal sleep study that indicated severe pediatric obstructive sleep apnea. He required jaw distraction surgery. Ultimately he continued to have respiratory difficulty including apnea due to obstruction which led to tracheostomy and home ventilator use. He was diagnosed with epilepsy at 9 months old. Seizure types have changed over time, requiring a combination of anti-epileptic drugs. Growth has been poor for weight, length, and OFC. At the age of 27 months his weight had somewhat improved some tracheostomy and was at 9%ile while length was at 2%ile. Analysis of growth hormone gave unremarkable results. He had a severe microcephaly with OFC, estimated -4.5 SD below the mean. He has had numerous respiratory infections requiring hospitalizations. He had pigment mosaicism of his skin of his extremities. He was not able not regulate body temperature well. He exhibited decreased sweating ability—cooling vest, portable fan, water misters were utilized frequently, even in indoor environment. He developed “hot spots” on his extremities near joints that resolved with no treatment. Scalp hair was very slow to grow in. He was born without eyebrows and eyelashes. Eyebrows have come in along with very few eyelashes over the years. Development was significantly impaired, he was rolling over and laughed when tickled. He had cortical visual impairment with optic disc atrophy. Primary teeth were erupting, some showed microdontia and abnormal shape. He had an idiopathic CD4+ T cell lymphocytopenia with 16 % (ref. 27-62%). He passed away after third birthday due to an infection of unknown origin. Trio-WES with DNA samples of both unaffected parents and the proband, revealed a *de novo* heterozygous single nucleotide substitution, c.1407G>T, in *BCL11B* (NM\_138576.3) resulting in a missense variant p.(Lys469Asn).

### Individual 24

This is a female born to unaffected, nonconsanguineous parents of European descent. Newborn screening was unremarkable but did not include an assessment of TRECs. She first developed seizures at around 3 months of age. She was observed to have low muscle tone and poor feeding due to difficulties with swallowing, which prompted both occupational and speech therapy, and necessitated NG tube placement. She was also noted to be hypermetropic and to have hypopituitarism with dysmorphic craniofacial features that included sparse hair, microcephaly, brachycephaly, a small anterior fontanelle, full cheeks, a wide nasal bridge with narrow nasal tip, hypoplastic alae nasi, a narrow mouth with downturned corners, thin lips. Brain imaging demonstrated an absent corpus callosum and simplified gyral patterns. She spent one month admitted to the neonatal intensive care unit,

during which time multiple evaluations were performed, including an echocardiogram, renal ultrasound, CSF studies, upper GI studies, and an EEG; these were unremarkable, as were high resolution chromosomes and a chromosomal microarray. She ultimately developed frequent respiratory infections, eczema involving the periorbital skin and scalp, and was reported to develop chicken pox following vaccination. She had persistent Idiopathic CD4<sup>+</sup> T cell lymphocytopenia (ICL) and poor T cell function, and 2 stem cell transplants failed. She passed away at 2 years of age from viral infections, respiratory syncytial virus and adenovirus, despite immunoglobulin replacement therapy. WES with DNA sample the proband, revealed a heterozygous single nucleotide substitution, c.1407G>C, in *BCL11B* (NM\_138576.3) resulting in a missense variant p.(Lys469Asn). Subsequent Sanger sequencing did not identify the *BCL11B* missense variant in parental DNA nor in DNA samples from three unaffected siblings.

### Individual 25

This is a 3-year-old female born to unaffected, consanguineous parents, first-degree cousins, from Egypt. Further family history is unremarkable. Her prenatal history was notable by gestational maternal diabetes and fetal ascites detected at 31 gestational weeks and which resolved spontaneously before birth. She was born at term, with no complications. Her birth weight was 3355 g (0.03 SD), birth length 49 cm (-0.95 SD) and birth OFC was 36.5 cm (1.46 SD). Apgar score was 9/10 at 1 minute and 10/10 at 5 minutes after birth. At birth she showed axial hypotonia, hypo reactivity, poor motility, with preserved crying and feeding, without myopathic signs. Following minor, dysmorphic facial features were observed, which included thin eyebrows, hypertelorism, flat and elongated philtrum, thin vermilion of the lips, and micrognathia. In the neonatal period she had cholestatic jaundice and was treated with phototherapy. Hyperferritinemia was documented but gradually decreased over time. Blood ammonium levels, urinary organic acid analysis and lysosomal acid lipase testing were all unremarkable. At 3 months, her head circumference increased over 97th percentile while the overall growth was unremarkable. At the age of 9 months she started to present symptoms of epilepsy with myoclonic manifestation and vigilance alteration together with further global neurological regression (especially increased day sleepiness), arising a suspicion of a genetically determined developmental and epileptic encephalopathy. At that time, at the neurological examination she presented profound developmental delay, being not able to reach eye contact (fixation deficit), nor to control the head or to grasp objects. She had hypotonia, tetraparesis, preserved but reduced deep tendon reflexes and spontaneous sporadic jerks. The electroencephalogram, performed at 10 months, showed poor organization with diffuse slow wave background and generalized spike-wave (around 2 Hz), sometime associated with palpebral myoclonus and brief ocular up gaze with features of myoclonic absences (Figure 1). Brain MRI showed signs of brain atrophy with enlargement of the peri-encephalic spaces, reduced opercularization in the frontal-temporal region, reduction of white matter, thinning of the corpus callosum, and abnormal rotation of the bilateral hippocampus. Visual evoked potential did not show cortical response. She was treated with valproic acid 30 mg/kg per day divided in two daily doses with partial improvement of reactivity and significant reduction of seizures. As seizure relapses occurred, therapy with ethosuximide and clonazepam was added. Recurrent episodes of increased transaminase have been documented, without other signs of liver involvement. In the suspicion of side effects to the anti-seizure medication, valproic acid and ethosuximide have been withdrawn, keeping on clonazepam, with partial improvement of hypertransaminasemia. Immunological evaluation did not detect any major immunologic

deficits. Exome next generation sequencing showed an heterozygous in the BCL11B gene: c.1414C>T, p.Arg472Cys. This variant was subsequently not detected in her parents.

### **Individual 26**

This is a 10 month old female of unaffected, nonconsanguineous parents. She was born at 39+2 weeks of gestation after an uneventful pregnancy. Body measurements at birth were unremarkable. Breast feeding was not possible due to severe muscular hypotonia. In the first months gingival hyperplasia and multiple gingival cysts were diagnosed. She also showed craniofacial abnormalities such as prominent forehead and deep set eyes. Brain MRI and EEG showed no abnormalities. At first clinical examination at our department, at 10 months she was not able to hold her head, roll over, to grasp and showed problems with coordination. The body measurements were: weight 7,2 kg (-1,66 SD), length 72 cm (+0,61 SD), OFC 44 cm (-1,0 SD). The child showed central muscular hypotonia and hypertonia of extremities. She showed dysmorphic features such as midface hypoplasia, frontal bossing, high narrow and small teeth. Karyotyping and Array CGH were unremarkable. Trio-WES with DNA samples of both unaffected parents and the proband, revealed a *de novo* heterozygous single nucleotide substitution, c.2421C>G, in *BCL11B* (NM\_138576.3) resulting in a missense variant p.(Asn807Lys).

### **Individuals 27-30**

This is a family with four affected family members, a father and his three sons. Individuals 27 and 28 are products of the first pregnancy of nonconsanguineous, Caucasian parents. Pregnancy was an uncomplicated twin gestation (zygosity undetermined at delivery, but likely monozygotic based on genome studies). Delivery via Caesarean section was at 39 weeks of gestation

### **Individual 27**

This is a 14-year old male proband. He had a birth weight of 3430g (-0.1 SD). He started walking at 13-14 months but often walked on his toes. He never ran or jumped. By age 6 years abnormal gait was noted, and about a year later (~age 7) he began walking on the side of his left foot. Symptoms progressed with posturing of his left hand behind his back during ambulation and progressive development of equinovarus deformity of the left foot. By age 10 years, the left foot was fixed in plantar flexion and could not be dorsiflexed with passive movements. Around age 11 years he developed dystonic and hyperkinetic movements, primarily in the arms, which seem to worsen with stress and fatigue. On exam at age 11, he had variable and fluctuating increased tone in his upper and lower extremities, with difficulty in performing smooth flexion or extension movements at the wrists / elbows, knees and ankle joints. He was characterized as having chronic, variable but overall progressive, focal with subsequent hemi dystonia and progression to likely generalized dystonia (trunk appears involved) without other evidence of neurologic dysfunction or parkinsonism. While there were not concerns about acquisition of fine motor milestones, he had long term difficulty with fine motor skills such as manipulating with Lego blocks, buttoning pants, and handwriting and required occupational therapy. He required surgical extraction of some deciduous teeth. He is described as a messy eater, and he did not use a fork for self-feeding until age 7 years and required feeding therapy. Speech development was delayed, and communication was primarily non-verbal between the ages of 2 and 4 years. He began speech therapy at age 4-5 years and this is ongoing. There are concerns about auditory

processing deficits. His level of neurocognitive functioning is broadly intact, with a few specific areas of weakness; he does well in a homeschool program with academic achievement at or above grade level. He has sensory aversions, poor eye contact, and lack of interest in peers. He prefers to interact with his twin brother. He has additional diagnoses of anxiety, obsessive-compulsive disorder /tic disorder. Recently he presented with significant anger outbursts. He is not diagnosed with Autism spectrum disorder, but this continues to be a diagnostic consideration. There are no cognitive concerns and he performs at or above expected grade level in academic subjects. Recently started Lexapro. At the age of 14 years, he is maintained on Trihexyphenidyl with baclofen to treat generalized dystonia. He ambulates unassisted w/ leaning to the right. Running improvement with slight dystonic flexion of the right leg at the knee. With tandem gait, he is unable to have heel touch his toe, and instead, his legs scissor. He continues to have trouble with handwriting. He continues to participate in regular speech therapy as speech assessment at age 14 years noted severe articulation disorder, mild dysfluency and pragmatic language deficits. Electromyogram and nerve conduction studies, neuro-ophthalmic examination, brain MRI, and screening for metabolic diseases associated with dystonia (eg creatinine synthesis/transport defects, Wilson disease, homocystinuria, etc) all gave unremarkable results. Spine imaging revealed presence of mild thoracic syrinx, with no evidence for tethered spinal cord or Chiari malformation. He was reported to have had frequent viral illnesses and environmental allergies.

Chromosome microarray, NGS Panel (*PNKD*, *PRRT2*) and *SLC2A1* del/dup panel – negative, a gene panel for genes associate with isolated dystonia (*TOR1A* deletion testing, *THAP1* sequencing) all gave unremarkable results.

#### **Individual 28 (twin of individual 27)**

This is a 14-year old male proband. He had a birth weight of 3799g (+0.7 SD). He started walking at 13-14 months but often walked on his toes. Could run, but never quickly. Has always had difficulty with fine motor skills. He met language milestones on time, and had more typical speech development as compared to his twin brother (individual 21). He began speech therapy around the age of 5 years secondary to concerns about articulation, fluency, and stuttering. Around his 12<sup>th</sup> birthday, the family noted new onset of a gait abnormality and increased difficulty with fine motor activities. On initial neurology clinical exam at 12 years, 8 months, right foot eversion and hyperkinesia were noted. Assessment at the age of 13 years by occupational therapy noted performance deficits in the areas of social interaction, fine motor coordination skills, bilateral coordination skills, visual motor skills, self-care skills, and higher level activities of daily living. At the age of 14 years, he is maintained on Trihexyphenidyl with baclofen to treat generalized dystonia. On assessment, his routine gait and running somewhat slow and awkward and he uses a handrail to help with tandem gait. He is hyperkinetic, has trouble holding still, with almost constant movements, sometimes choreiform in appearance. When writing, he has very awkward pencil grip; writing is very awkward and he lifts up right shoulder, straightens out arm, repeatedly stops, moves away, then starts again- dystonic posturing of the right wrist. This is a daily occurrence, every time with this activity. Is able to play piano (it is a challenge). The medical care team is considering botox injections for his right arm. He required surgical extraction of some deciduous teeth. He continues to participate in regular speech therapy as speech assessment at age 14 years noted that he has severe articulation disorder and, mild stuttering, cluttering. There are concerns about auditory processing deficits. Similar to his brother, he has additional diagnoses of anxiety, obsessive-compulsive disorder that interfere with day to day activities. He is not diagnosed with Autism, but this continues to be a diagnostic consideration. There are no cognitive concerns and he performs at or above

expected grade level in academic subjects. Recently started Lexapro. Like his brother he is described as a messy eater, due to poor fine motor skills. He was reported to have had frequent viral illnesses and environmental allergies.

#### **Individual 29 (younger brother of twins 27 and 28)**

This is an 8-year old male, born at 39 weeks of gestation. His birth weight of 4054g (+1.3 SD). He had unremarkable acquisition of gross and fine motor skills as well as of speech and language milestones. He began speech therapy at the age of 3 years due to articulation issues. As he got older he was noted to "gallop" when he ran. By the age of 4 years he started running with a limp and complained of left knee and leg pain. Symptoms worsened significantly over a few months around the age of 5 years, to include periods of severe rigidity dystonia with pain, especially in the lower extremities. Initially dystonia was episodic, with symptoms lasting from a few hours to all day. Symptoms then progressed to very severe pain present 5-6 days per week. He had dramatic improvement from Trihexyphenidyl (Artane) within a week of initiation of this medication, and was discharged from physical therapy intervention 4 months later with full ability to participate in age activities including jumping, running, galloping, transfers, and stair ambulation with symmetrical weight shifting over bilateral lower extremities. Ataxic quality, abnormal muscle tone was observed only when confined or stressed. At the age of 8 years, he continues on Trihexyphenidyl with added high dose baclofen to treat generalized dystonia and is generally able to participate in physical activities that require running. He keeps his torso flexed forward when he runs, resulting in a crouched appearance. The family reports that he sometimes presents with limping and complaints of pain. He does feel that the most pain is when he is walking, but he does not feel the pain as often (no longer present daily). Routine gait shows circumduction of left leg. He has trouble lifting left heel up when he walks, pointing toe. He leans/drifts to the right when sitting unsupported and standing. While seated there is still a tendency for his legs to scissor with extension. Fine finger movements impaired left > right. Still has slight extension of the neck (retrocollis) and torso during upper extremity motor tasks but improved. The family describes some withdrawal/wearing-off symptoms between doses of Trihexyphenidyl. He continues to receive speech therapy at school. There are concerns about auditory processing deficits. He also suffers from frequent vomiting. He was reported to have had frequent viral illnesses but no history of allergies or asthma.

#### **Individual 30 (father of individuals 27-29)**

This is a 40-year-old male, father of individuals 27-29. At time of family ascertainment, he was reported a history of toe walking, tight heel cords and difficulties with fine motor tasks. He prefers to walk with wedge orthosis, and will have cramping with prolonged standing (worse if not using his orthoses). After identification of the familial *BCL11B* variant, he further reported that as a child he often had viral infections resulting in high fever, and was described as clumsy. He states that he has devised strategies to cope with routine fine motor skills required in adulthood, but he often struggles with novel or infrequent fine motor tasks. He is often in motion and is by nature somewhat hyperkinetic; if he must remain unmoving or still for extended periods of time, this requires conscious control. He has never been described or diagnosed with autism spectrum disorder or has having autistic features. Cognitively, he has no concerns. He has a post baccalaureate degree (masters of business administration) and acts in an independent leadership role at his place of employment. He has no history of asthma but is reported to have environmental allergies and adverse reactions to multiple medications. He was diagnosed with Henoch-Schonlein purpura in adulthood.

Quint genome sequencing of the DNA samples of the three affected brother, their affected father and unaffected mother identified a heterozygous single nucleotide substitution, c.2422T>C, in *BCL11B* (NM\_138576.3) resulting in a missense variant p.(Cys808Arg) that was present in all 4 affected individuals. Subsequent targeted analysis did not identify this variant in parental DNA's of individual 30, suggesting *de novo* occurrence.

Additionally, both twins had a *de novo* variant c.562G>A, p.(Glu188Lys) in *ADAR* (NM\_001111) and a maternally inherited variant c.1808T>C, p.(Met603Thr) in *POLG* (NM\_001126131) both of which are classified as variants of uncertain significance according to ACMG guidelines, and not present in DNA samples of individuals 29 and 30. Moreover, all three brothers (individuals 27-29) were hemizygous for a maternally inherited variant c.883G>A, p.(Gly295Ser) in *SRPX2* (NM\_014467) that is also classified as variant of uncertain significance according to ACMG guidelines.

### Individual 31

This is a 12-year-old female, the first child of unaffected, non-consanguineous parents. She was born at 39 weeks of gestation after an uneventful pregnancy. Muscular hypotonia was noticed in the first months of life and head holding was delayed. Sitting was reported at 12 months and free walking at 18 months of age with an unstable gait and tiptoe walking. At the age of 3 years she showed a typical spastic gait pattern, the lower extremities are more affected than the upper extremities. Treatment with botox injections and dopamine showed a worsening of the gait pattern. Under febrile infections motor skills deteriorated without complete recovering after the event. Language development was delayed, with no words spoken at two years of age. Further on the girl developed a dysphonia and dysarthria. The cognitive development was within average range (IQ 98). Due to dental anomalies of the incisors two operative correction were performed, an oropharyngeal dysphagia was not present. At the last clinical examination her weight was 66 kg (+1.0 SD), height was 163 cm (+1.62 SD) and OFC was 58 cm (+3.20 SD). Dysmorphic features included prominent nose, long philtrum, thin upper lip vermilion and hypertelorism. She was able to walk but need a wheelchair for longer distances. Metabolic and routine biochemical tests as well as brain and spine MRI were unremarkable. Karyotyping and SNP-array analysis showed unremarkable results. Trio-WES with DNA samples of both unaffected parents and the proband, revealed a *de novo* heterozygous single nucleotide substitution, c.2507G>A, in *BCL11B* (NM\_138576.3) resulting in a missense variant p.(Ser836Asn).

### Individual 32

This is a 17-year-old male, second of three children of unaffected, nonconsanguineous parents. The first pregnancy of the couple ended in fetal death at 4 months of gestation. Further family history was unremarkable. He was born at term after an uneventful pregnancy. Birth weight was 3900 g, (0.96 SD) birth length was 51 cm (-0.39 SD) and OFC was 34.5 cm (-0.69 SD). The APGAR was 10. In the first few months he had plagiocephaly, cow's milk protein intolerance and recurrent bronchitis. He sat up at 9 months and walked at 18 months of age. Thereafter, he had a severe delay in language; he did not speak until the age of 3 years. He displayed learning difficulties and attended a special school from the age of 6 years. At the age of 12 years, he was treated for acute T-cell lymphoblastic leukemia, non-hyper-leukocytic without neuro-meningeal involvement and with a mediastinal tumour syndrome. At the same time he developed hypertonia of the lower limbs, ataxia and hollow feet probably favoured by the chemotherapy treatment with vincristine. At the age of 14 years, he had good comprehension skills

but said only a few words. At 17 years, his weight was 39.4 kg (-4SD); his height was 164 cm (-1,5 SD) and OFC was 54 cm (-0,5 DS). Physical examination showed prominent nose, long philtrum, thin upper lip vermillion, high-arched palate, bifid uvula, large protruding ears and retrognathism. He was in complete remission for leukaemia 2.5 years after completing treatment. Fragile X study, karyotyping and array- CGH array all gave unremarkable results. Trio-WES with DNA samples of both unaffected parents and the proband, revealed a *de novo* heterozygous single nucleotide substitution, c.2507G>A, in *BCL11B* (NM\_138576.3) resulting in a missense variant p.(Ser836Asn).

### Individual 33

This is a 5-year-old male, the first child of unaffected, nonconsanguineous parents. The pregnancy was complicated by a subchorionic bleeding at 6 weeks of gestation as well as maternal hypertension. He was delivered by emergency caesarean section at 39 weeks gestation, due to a non-reassuring stress test. His birth weight was 2435g (-2.4 SD), birth length was 49 cm (-1.2 SD), and head circumference was 31.5cm (-2.9 SD), with APGAR scores were 9 and 9, after first and fifth minute respectively. After birth, he was noted to have a cleft palate, large and prominent ears, and dysmorphic facial features. He stayed in hospital after birth for 16 days, related to issues with poor feeding. An echocardiogram was completed and revealed a small atrial septal defect. This infant had a positive newborn screening result for severe combined immune deficiency, with no detectable TRECs (T cell receptor excision circles). Follow-up testing revealed pan-lymphopenia, hypogammaglobulinemia, abnormal T cell receptor diversity, decreased naïve T cells, and abnormal T cell proliferation to mitogens. The family history is non-contributory, other than the mother having also had large and prominent ears at birth, for which she later went on to have cosmetic surgery. Global developmental delay was apparent from infancy. At the age of 5 years, he cannot sit independently, is non-ambulatory and non-verbal. He has central hypotonia with significant peripheral hypertonia and spasticity, cortical vision loss, and intractable epilepsy. MRI of the brain completed at 4 months of age revealed dysgenesis of the corpus callosum, dilatation of the bilateral trigones and occipital horns and enlargement of the third ventricle. He has eczema, constipation and is now G-tube fed, due to ongoing issues with feeding and swallowing. He has had two episodes of aspiration pneumonia treated with IV antibiotics and stepped down to oral therapy to complete the course, and both episodes were responsive to treatment. There have been no other clinically significant infections. At his last clinical examination at the age of 3 years and 4 months, his height was 89.5cm (-2.5 SD), and his weight was 11.7 kg (-2.2 SD). He has myopathic facies, long and upslanting palpebral fissures, a prominent nose with low columella, large and protruding ears with a simplified external ear, a long philtrum and thin upper lip vermillion. Array-CGH analysis did not reveal any pathogenic copy number variants. Trio-WES with DNA samples of both unaffected parents and the proband, revealed a *de novo* heterozygous single nucleotide substitution, c.2513A>C, in *BCL11B* (NM\_138576.3) resulting in a missense variant p.(Lys838Thr). In addition, a *de novo* heterozygous variant c.982T>A, p.(Phe328Ile) was identified in *CCR7* (NM\_001838) its clinical significance remains unknown.

### Individual 34

This is a 24-year-old male, the first born child of unaffected, non-consanguineous parents. He was born after an uneventful pregnancy at term. His birth weight and length were unknown. He was diagnosed with spastic cerebral palsy at age 1. He had global developmental delays and intellectual disability during childhood, and his mother reported significant regression during his teenage years. He had scoliosis surgery during childhood. During childhood, he had frequent infections, but since leaving school his health has been stable. Currently, at the age of 24, he is non-ambulatory and has no speech. Further, he has abnormal involuntary movements, muscle weakness, static encephalopathy, and muscle contractures. He has overfolded ears, narrow palate, fingers and left thumb hypertrophy, lower greater than upper limbs. EEG gave unremarkable results. He is followed for idiopathic thrombocytopenic purpura and by cardiology for aortic ectasia and. Trio-WES with DNA samples of both unaffected parents and the proband, revealed a *de novo* heterozygous single nucleotide substitution, c.2513A>G, in *BCL11B* (NM\_138576.3) resulting in a missense variant p.(Lys838Arg). In addition, the proband also harbored a biallelic pair of variants in *SYNE1*, a heterozygous variant of uncertain significance and a heterozygous *LPATH* variant.

### Individual 35

This is a 15-year-old male, born to unaffected, nonconsanguineous parents after an uncomplicated pregnancy. His early development was unremarkable, he walked independently at 11 months. As a toddler he used to bang his head to the floor out of frustration. Around 4-5 years of age his motor development deteriorated, and he was diagnosed with developmental coordination disorder (DCD). At the age 6 years, when he was learning to write, difficulties with fine motor skills were noted. "Shaking movements" started around 10 years of age. These movements are interfering with daily activities like holding a glass or writing. These movements were later classified as probable subcortical myoclonia. Neuropsychological testing at age 12 years showed mild intellectual disability with vIQ 80 and pIQ 60. At 9 years of age was diagnosed with ADHD requiring medication. His behavior is challenging, and he has regular temper tantrums where he is out of control. On several occasions he was placed in care for long periods. He has no history of recurrent infections or allergies, and has unremarkable vision and hearing. At the age of 13 years his height was 162 cm (-0.5 SD), weight was 40.4 kg (-1.5 SD), and OFC 53.5 cm (-1.5 SD). At physical examination he was friendly and cooperative. He has a triangular face, plagiocephaly, thin eyebrows, upslanted palpebral fissures, high nasal bridge, mild retrognathia, prominent antihelices of the ears. He had a subtle pectus excavatum. He had very frequent myoclonia of the extremities, and in lesser extent the trunk and head, with unremarkable coordination. Brain MRI, EEG, as well as metabolic screen in urine and plasma gave unremarkable results. SNP-array analysis identified no copy-number variants. WES with DNA sample of the proband revealed a heterozygous single nucleotide substitution, c.2519C>T, in *BCL11B* (NM\_138576.3) resulting in a missense variant p.(Thr840Met). Subsequent Sanger sequencing did not identify the *BCL11B* missense variant in parental DNA.

### Individual 36

This is 5-year-old male, born at 38 weeks of gestation to unaffected, nonconsanguineous parents. His birth weight was 3643g +0.8 (SD), birth length was 48.26cm (-1.2 SD), and OFC was not recorded. He was noted to be

hypotonic at birth. He was diagnosed with laryngomalacia in infancy and underwent supraglottoplasty. Facial dysmorphisms include long face, hypnotic appearance, broad and flat nasal bridge, prominent forehead, epicanthal folds with small palpebral fissures, pseudoesotropia, microretrognathia. Mother noted developmental delays in all areas. Motor skills were delayed with rolling at 5 months and walking at 18 months. Gross motor skills improved over time with independent walking but unsteady runny by age 3. He had more prominent expressive and receptive language delay. By age 3 he was using a few words but spoke in his own language. He was diagnosed with autism spectrum disorder, and was enrolled in physical therapy, occupational therapy, and speech therapy. Mild eczema was diagnosed at 12 months. Food allergies to peanut and tree nuts developed at age 3 years. Asthma was diagnosed at age 5 years following an exacerbation. Currently well controlled. Immunologic work-up identified mild decreases in total CD3, CD4, and CD8 T cell counts with unremarkable IgG level. He does not have a significant history of infections. Trio-WES with DNA samples of both unaffected parents and the proband, revealed a *de novo* heterozygous single nucleotide substitution, c.2522G>T, in BCL11B (NM\_138576.3) resulting in a missense variant p.(Arg841Leu).

### Individual 37

This is an 8-year-old female, the first child of unaffected, nonconsanguineous parents. The pregnancy was uneventful and she was born at 40 3/7 weeks of gestation via vacuum assisted delivery following induction. She had a birth weight of 3583 g (+ 0.75 SD), length of 54.61 cm (+ 0.46 SD), and OFC was not recorded. The neonatal period was complicated by jaundice requiring hospitalization for phototherapy in addition to poor feeding with gastroesophageal reflux requiring an elemental formula. The family history is non-contributory. Developmental milestones were as follows: she was able to sit unsupported at 6 months, crawled at 10 months and walked at 15.5 months, single words at 14 months. She has lingual dyspraxia. Behavioural concerns including attention deficits, hyperactivity, rigidity related to routines, and outbursts with prolonged crying which persists to age 8 years at the most recent evaluation. Neurocognitive testing revealed functioning in the low to extremely low range. Last examination at age 8 years she was pleasant with immature vocabulary, speech pattern, and behaviour. Examination revealed facial hypotonia with drooling, plagiocephaly, ears with overfolded helices and two café au lait spots, small teeth, otherwise generally non-dysmorphic. Her height was 128 cm (- 0.45 SD), weight 28 kg (- 0.11 SD), OFC 53 cm (+ 0.88 SD). Feeding difficulty continued through early childhood culminating in the diagnosis of eosinophilic esophagitis. She has mild intermittent asthma and mild hyperopia. Brain MRI was unremarkable. SNP-array identified no copy-number variants. Trio-WES with DNA samples of both unaffected parents and the proband, revealed a *de novo* heterozygous single nucleotide substitution, c.2525A>C, in BCL11B (NM\_138576.3) resulting in a missense variant p.(His842Pro).

### Individual 38

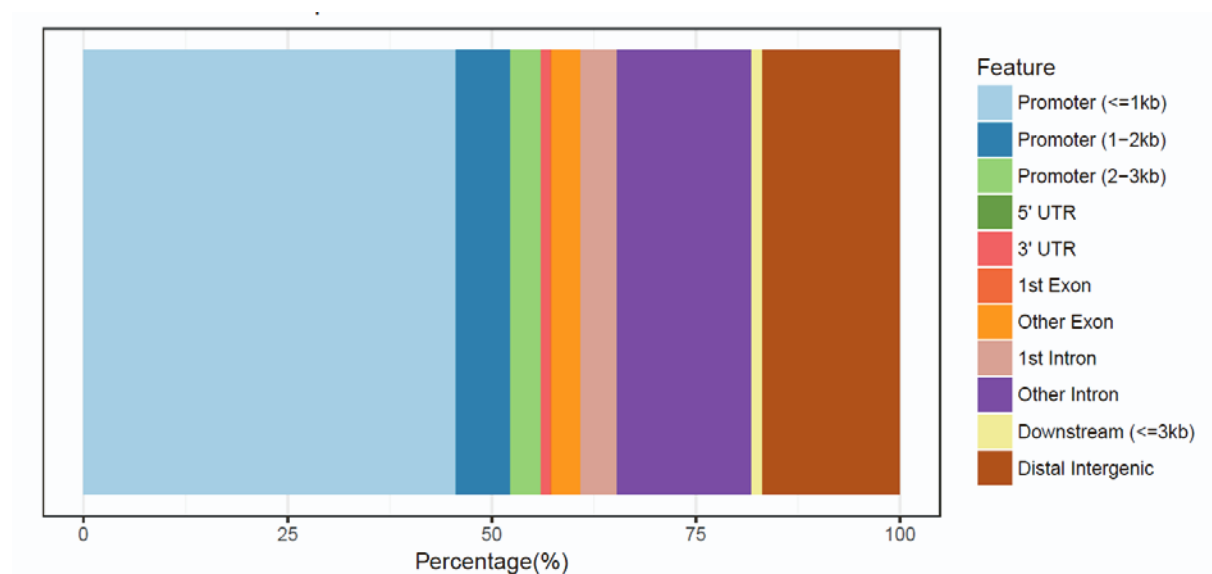
This is a 6-year-old male, the second child of unaffected non-consanguineous parents. His older brother has a history of speech delay, further family history was non-contributory. He was born at full term by vaginal delivery after uncomplicated pregnancy. His birth weight was 2900g. Postnatal period was characterized by chronic otitis media and pneumonia, and he failed at hearing screening. Motor developmental was mildly delayed, he sat at 12

months, started to walk at 18 months. He spoke first words at 18 months, and developed a speech delay with only 3-4 words at 4 years of age. At last clinical examination, at 6 years and 4 months, his weight was at the 48 percentile, height was at the 38 percentile, and head circumference in the 99 percentile. Physical exam showed frontal bossing, hypertelorism, mild exophthalmos. He was non-verbal, followed mother commands but had a poor eye contact. He was diagnosed with autism spectrum disorder. Ophthalmologic exam at 3 years of age revealed a physiologic hyperopia. He was reported to have eosinophilia, and has asthma and multiple food allergies including peanuts, milk, eggs and wheat. MRI of the brain showed two arachnoid cysts. SNP-array revealed no copy number variations and a single 10.3Mb ROH on the short arm of the chromosome 8. Trio-WES with DNA samples of both unaffected parents and the proband, revealed a *de novo* heterozygous single nucleotide substitution, c.2536C>G, in *BCL11B* (NM\_138576.3) resulting in a missense variant p.(His846Asp).

### Individual 39

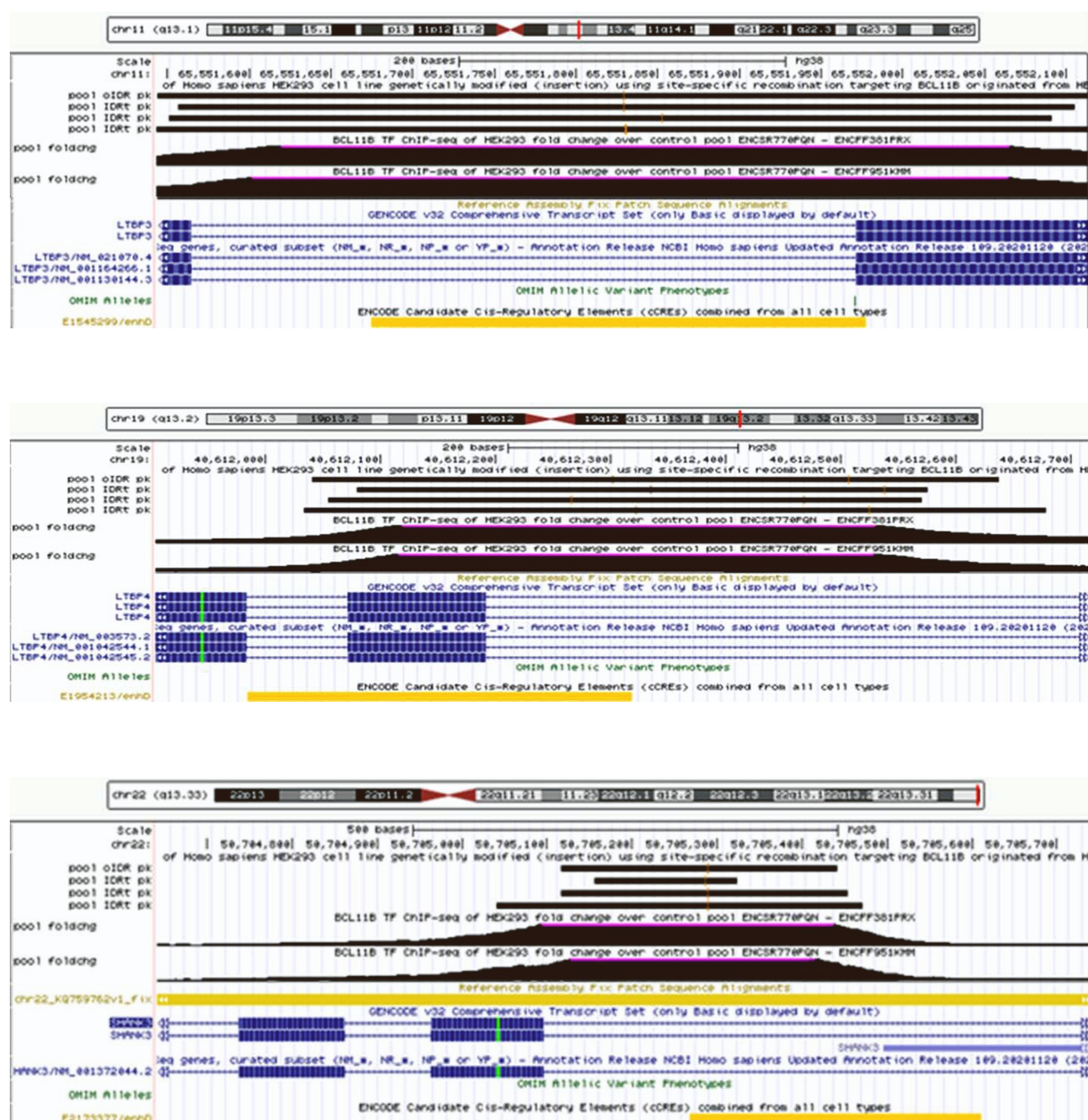
This is an 8-year-old male, referred to Clinical Genetics for learning issues, behavior problems and a diagnosis of autism spectrum disorder with subtle dysmorphic features. He was born at full-term with birth weight of 3175g. There was early failure to thrive and feeding difficulty, with hypotonia, delayed motor milestones and speech delays. He received diagnosis of autism spectrum disorder at around age of 4 years. He was very rigid, he was obsessed with wanting real tools not toy tools, he had to wear the same shirt and shoes every day, and he was lining up cars and opening and closing doors. As he got older his expressive language improved, and he does not meet official criteria for autism. He continues to receive special education services with speech therapy, physical therapy and Applied Behavior Analysis therapy. He has always been very impulsive and aggressive, hitting his parents and having issues in school with behavior. He has poor emotional regulation. Impulsivity has expressed itself as lying, taking things that do not belong to him as well as aggression. He was small always. He often develops rash around the mouth from drooling/sucking on finger. Family history was negative for any relevant concerns. MRI at age 1 year showed subtle nonspecific T2 hyperintensities in the region of the insula and extreme capsules bilaterally. Echocardiogram was unremarkable. Exam at age 8 years revealed: Height: 115.5 cm Percentile: <1 %ile (Z= -2.81) Weight: 18.90 kgs Percentile: <1 %ile (Z= -3.05), Head circumference: 48cm Percentile: <2 %ile (Z <-2.05). Dysmorphic features noted were thin and arched eyebrows, short, upslanted palpebral fissures with narrow opening, smooth philtrum and thin upper lip. Palmar creases were abnormal on one hand. He had generalized hypotonia with no focal deficits or abnormal movements. Whole genome chromosomal microarray was negative for copy number changes of significance, areas of homozygosity or uniparental disomy. Trio-WES with DNA samples of both unaffected parents and the proband, revealed a *de novo*, heterozygous single nucleotide substitution, c.2629 C>T, in *BCL11B* (NM\_138576.3) resulting in a missense variant p.(His877Tyr)

**Figure S1. Analysis of Chromatin ImmunoPrecipitation DNA-Sequencing (ChIP-seq) dataset**



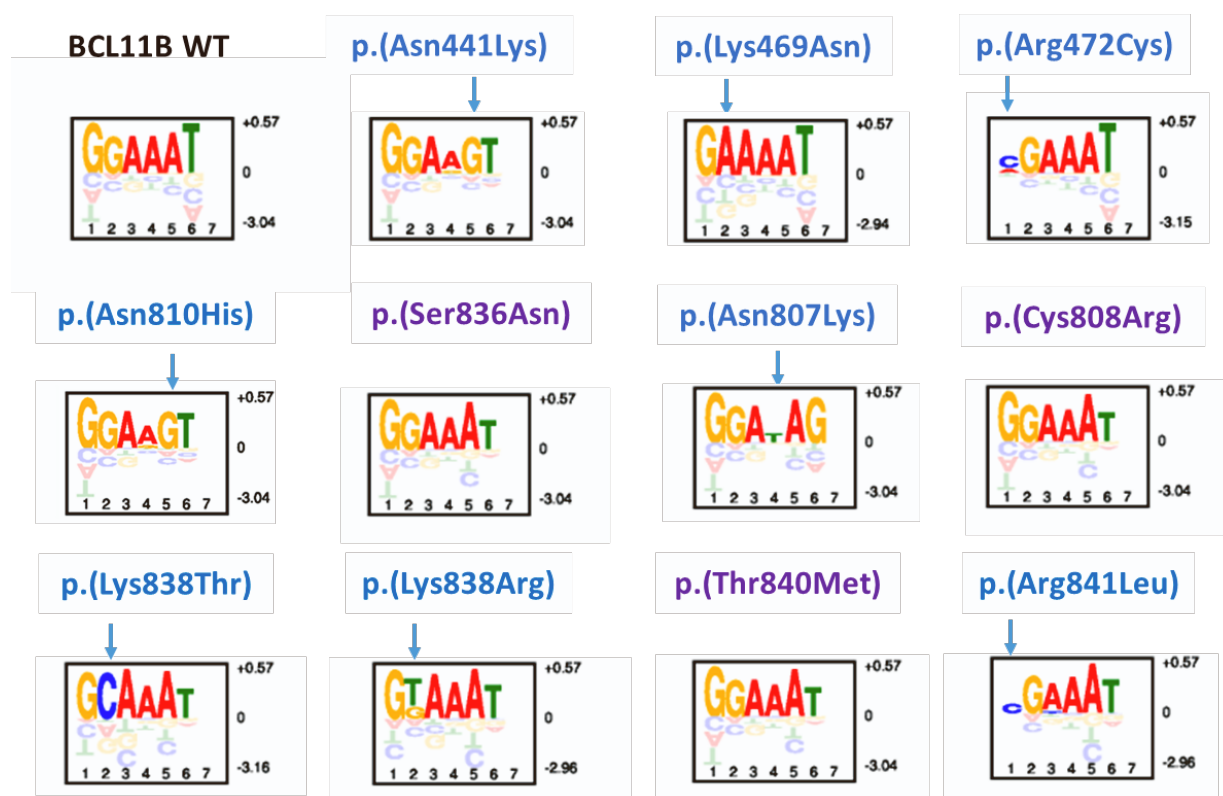
Analysis of Chromatin ImmunoPrecipitation DNA-Sequencing (ChIP-seq) dataset obtained in HEK293 cells (accession ENCSR770PQN), generated by the ENCODE project. Shown is the distribution of all conservative peaks.

**Figure S2. Position of BCL11B binding sites at the *LTBP3*, *LTBP4* and *SHANK3* loci**



Shows the position of BCL11B binding sites at the *LTBP3*, *LTBP4* and *SHANK3* loci. Chromatin ImmunoPrecipitation DNA-Sequencing (ChIP-seq) dataset obtained in HEK293 cells (accession ENCSR770PQN), generated by the ENCODE project.

**Figure S3. Predicted DNA binding motif for the BCL11B-WT and each of the missense variants within the  $\alpha$ -helix of the C2H2-ZnF domains**



Shows predicted DNA binding motif for the BCL11B-WT and each of the missense variants within the  $\alpha$ -helix of the C2H2-ZnF domains. Note that all missense variants affecting a “specificity residue” (shown in blue, differences are marked with an arrow) alter the DNA binding site and are therefore predicted to bind to different alternative genomic sequences as compared to the BCL11B-WT. Missense variants within the  $\alpha$ -helix, not affecting a “specificity residue” (shown in violet) do not alter the DNA binding specificity. Data are obtained by “Zinc finger recognition code”, Najafabadi *et al.* 2015.