CUX1-related neurodevelopmental disorder: Deep insights into phenotype-genotype spectrum and underlying pathology

Henry Oppermann  
*University of Leipzig*

Christina A Gurnett  
*Washington University School of Medicine in St. Louis*  
et al.

Follow this and additional works at: [https://digitalcommons.wustl.edu/oa_4](https://digitalcommons.wustl.edu/oa_4)

Part of the *Medicine and Health Sciences Commons*

**Recommended Citation**

[https://digitalcommons.wustl.edu/oa_4/3559](https://digitalcommons.wustl.edu/oa_4/3559)
CUX1-related neurodevelopmental disorder: deep insights into phenotype-genotype spectrum and underlying pathology

Heterozygous, pathogenic CUX1 variants are associated with global developmental delay or intellectual disability. This study delineates the clinical presentation in an extended cohort and investigates the molecular mechanism underlying the disorder in a Cux1+/− mouse model. Through international collaboration, we assembled the phenotypic and molecular information for 34 individuals (23 unpublished individuals). We analyze brain CUX1 expression and susceptibility to epilepsy in Cux1+/− mice. We describe 34 individuals, from which 30 were unrelated, with 26 different null and four missense variants. The leading symptoms were mild to moderate delayed speech and motor development and borderline to moderate intellectual disability. Additional symptoms included microcephaly, developmental delay, seizures, and autism spectrum disorder. Heterozygous CUX1 variants result in a non-syndromic phenotype of developmental delay and intellectual disability. In some individuals, this phenotype ameliorates with age, resulting in a clinical catch-up and normal IQ in adulthood. The phenotype-genotype spectrum and underlying pathology are highly variable, and some subpopulations of hippocampal neurons express CUX1 [5, 6]. CUX1 active enhancers contain human accelerated regions. These regions are conserved across most mammals but highly divergent in humans that might have contributed to acquiring human traits such as cognition during evolution [7]. In agreement, variants in the CUX1-HAR region link to autism spectrum disorder (ASD) [7, 8].

In mice, CUX1 expression also decreases with age, whereas in early postnatal animals significantly more than in adults. In summary, disease-causing CUX1 variants result in a non-syndromic phenotype of developmental delay and intellectual disability. In some individuals, this phenotype ameliorates with age, resulting in a clinical catch-up and normal IQ in adulthood. The post-transcriptional balance of CUX1 expression in the heterozygous brain at late developmental stages appears important for this favorable clinical course.

European Journal of Human Genetics (2023) 31:1251–1260; https://doi.org/10.1038/s41431-023-01445-2

INTRODUCTION

CUX1 is a conserved mammalian homolog of Drosophila melanogaster Cut that encodes for two types of proteins through alternative splicing: cut-like homeobox 1 (CUX1) and cut alternately spliced protein (CASP) [1]. CUX1 isoforms are transcription factors, whereas CASP are Golgi proteins that share with CUX1 the N-terminal region but not the DNA binding motifs [2]. CUX1 proteins comprise long and short isoforms. The full-length p200 isoform contains four DNA binding motifs, three CUT repeats, and a homeodomain. It is proteolytically cleaved into two shorter isoforms during the cell cycle. Additionally, alternative splicing generates several other shorter CUX1 isoforms [1]. p200 CUX1 acts as a transcriptional repressor, whereas short CUX1 isoforms can activate or suppress transcription [1, 3].

In mammals, many tissues express CUX1/Cut1, including the developing and mature central nervous system [4]. In the human brain, most pyramidal neurons in layers (L) 2–5 of the cortex and some subpopulations of hippocampal neurons express CUX1 [5, 6]. CUX1 active enhancers contain human accelerated regions. These regions are conserved across most mammals but highly divergent in humans that might have contributed to acquiring human traits such as cognition during evolution [7]. In agreement, variants in the CUX1-HAR region link to autism spectrum disorder (ASD) [7, 8]. In mice, CUX1 expression also defines mature and young pyramidal neurons of L2/3 and L4 (known as the upper layers), regulating the formation of dendritic arbors and callosal connections during development [9–11].

While all of the above support crucial roles of CUX1/Cut1 in human neurodevelopment, these are only partially understood. Most known CUX1 functions derive from studies on cancer cells, where the gene can act as an oncogene or a haploinsufficient tumor suppressor gene [12]. Studies in mice have struggled with the lethality of Cux1 null variants. Homozygous knockout mutants (Cux1−/−) died shortly after birth due to underdeveloped lungs.
and respiratory failure [13], and only in outbred genetic back-
grounds some mice survived to adulthood. These animals showed
delayed growth and abnormal hair but no gross neurological
symptoms [8]. Cux1 heterozygous (Cux1+/−) animals are fertile and
display no obvious phenotypes, but an in-depth analysis of the
consequence of heterozygosis has never been pursued [13].
We previously reported nine individuals with heterozygous
truncating variants in CUX1. All individuals had speech delay and
most individuals exhibited motor delay and intellectual disability
(ID). Furthermore, some individuals seemed to catch-up on their
speech and motor development [14]. Notably, this study did not
address the mechanisms underlying the haploinsufficiency of
CUX1 in neurodevelopment.

Here, we review 11 previously [14–16] reported and 23
unpublished individuals with rare heterozygous CUX1 variants,
refining the associated clinical phenotype. Furthermore, we
investigated Cux1 expression and the phenotype of Cux1+/− mice
pertinent to the clinical phenotype of the individuals.

MATERIAL AND METHODS

Study subjects

Through matchmaking and collaborative efforts [17, 18], we identified 23
previously unreported individuals with CUX1 variants. Phenotype and
genetic information was obtained from the referring physicians via a
standardized questionnaire, together with brain MRI images. The descrip-
tion of the severity of the developmental delay and ID is based on IQ tests
(based on the experience of each center, some tests are suitable for
individuals younger than 6 years of age) and, if these were not available or
not possible, on an age-appropriate assessment by the attending
physician. Variants were identified using trio/single exome or genome
sequencing, or chromosome microarray. When needed, we performed a
segmentation analysis using Sanger sequencing. We also included the
information of eleven previously published individuals harboring hetero-
ygous truncating or de novo missense CUX1 variants [14–16]. For
individual 25 we have collected further information. If not described
otherwise, frequencies of clinical symptoms are described as the number
of affected vs. assessed individuals. Variants were mapped to CUX1
(NM_001202543.2) and CASP (NM_001913.4) GRCh37. We used the in silico
scores CADD [19], REVEL [20] an MutPred2 [21] to predict the impact of the
variants, including the individuals presented in this study, developmental
symptoms [8].

RESULTS

Immunohistochemistry and Nissl staining

Mice were anesthetized using an intraperitoneal (i.p.) injection of ketamine
and xylazine and perfused with formalin (Sigma). Brains were postfixed in
formalin, cryoprotected in 30% sucrose, and cut in 50 μm free-floating
cryosections. Sections were incubated with rabbit anti-Cux1 antibody
(Santa Cruz Biotechnology, M222X), goat anti-rabbit Alexa 488 (Thermo
Fisher Scientific, #A11034), and 4',6-diamidino-2-phenylindole dihydro-
dichloride (DAPI) (Merck, #D9542). Nissl staining was performed as previously described [26].

Confocal imaging, microscopy, and quantification

Images were taken using a Leica DM4B (Leica) with a 2.5X objective and
LAS AF v1.8 software (Leica; see Supplementary Materials for details).

Kainic acid model of epilepsy

10–13 week old mice were intraperitoneal injected with kainate (30 mg/kg
of body weight) (Milestone PharmTech USA), and they were monitored for
120 min by video recording. We used a modified version of the Racine
scale to score behavioral alterations [27]: 1-immobility, staring; 2-nrigidity
and automatisms; 3-unilateral forelimb clonus, forepaws on the belly; 4-
rearing; 5-continuous rearing and falling; 6-total body clonus. Stages 1 and
2 were considered non-convulsive seizures, while stages 3–6 represented
convulsive seizures. The maximum score of each animal and the latency
to onset of stage 3 were quantified.

Facial analysis

We performed the GestaltMatcher approach [24] on CUX1 individuals to
analyse the facial similarities among the nine individuals (Ind. 6, 7, 8, 9, 14,
18, 19, 25, and 28) who consented to the facial analysis (see Supplementary Materials for details).

Animals

We obtained Cux1+/− (Cux1Im2Opy) mice from A.J. van Wijnen (University of
Massachusetts Medical School, Worcester, MA) [13] and maintained in a
C57BL6J/RccHsd background (Envigo Laboratories, formerly Harlan, India-
napolis, USA). We used WT, Cux1−/−, and Cux1+/− littermates in all
experiments. The day of appearance of a vaginal plug was defined as E0
and the day of birth, postnatal day 0 (P0). Animals were housed and
maintained following the European Union Council Directive (86/609/
European Economic Community).

Western blotting

We analyzed the Cux1 p200 isoform expression in somatosensory cortex of
WT, Cux1−/− and Cux1+/− mice via Western blot by using antibodies
against CUX1 (Proteintech, 11733-1-AP; see Supplementary Materials for
details).

RT-qPCR analysis

Tissue dissection was performed as described above. cDNA synthesis and
qPCR reactions were performed as previously described [11, 25] (see
Supplementary Materials for details).
36 months, individual 25 presented with significantly delayed speech (spoke 5–10 words) and motor development, muscular hypotonia, and macrocephaly. He could speak in 2–3 word sentences at 42 months, with further speech improvements at 4.5 years of age. At 5 years of age, the speech was comparable to children of the same age. However, due to muscular hypotonia motor development was still markedly delayed. At the age of seven, the individual enrolled in a mainstream school, albeit with integration aids for motor difficulties. Formal testing revealed an IQ of 90. At age nine, he was able to participate fully in school sports. The other six individuals presented variable delay of speech development in their earlier examinations, reporting unremarkable speech at last exam – 10 words and motor development, muscular hypotonia, and macrocephaly. Eight individuals (8/26, 31%) developed seizures. The mean onset of seizures was 3 years and ranged between 1 and 6 years of age, with variable seizure types (including tonic-clonic and myoclonic seizures). All but two individuals became seizure-free (between 17 months and 22 years of age). Unfortunately, no further details regarding epilepsy type or EEG are available for the affected individuals. We could not observe a correlation between seizures and the severity of ID. Eight of the 20 individuals with available brain MRI imaging (performed and interpreted by each center) had no abnormalities. In the other 12 individuals, non-recurrent changes, such as a slightly prominent fourth ventricle, Chiari malformation, and white matter T2 hyperintensities, were observed (12/20, 60%; total 35%).

### Additional symptoms

Six individuals exhibited short stature (6/28, 21%), and eight showed joint laxity (8/20, 40%; total 23%). Abnormalities of the cardiovascular system, including persistent ductus arteriosus, atrial septal defect, and ventricular septal defect, were observed in eleven individuals (11/26, 42%; total 32%). Additional findings include mild scoliosis (3/21, 14%; total 9%) and genital malformations such as hypospadias, micropenis, and bilateral testicular ectopia in ten males (10/16, 63%; total 40%). The examined individuals had no apparent shared facial gestalt (Fig. S1). However, ten individuals displayed abnormalities in calvarial morphology, including macrocephaly, brachycephaly, plagiocephaly, and dolichocephaly. Eight individuals had a broad forehead (8/26, 31%), and four displayed frontal bossing (4/26, 15%). Seven...
individuals had low-set ears (7/25, 28%), and three had retrognathia (3/21, 14%). The physician's examination was also consistent with the facial analysis performed by GestaltMatcher [24]. Although only nine individuals consented for a facial analysis, they might share a similar facial phenotype on the cohort level, as they might share a certain degree of similarity, but some individuals presented heterogenous facial phenotypes.

We also identified two individuals with a de novo missense variant in CUX1 that affects only the transcript encoding CASP but not CUX1 (Table S1, Fig. 1). The first individual (CASP_1:c.1820T > C, p.(Met607Thr)), had congenital glaucoma and short stature but no neurological symptoms. In contrast, a second individual (CASP_2:c.1570C > T, p.(Arg524Cys))) had severe global developmental delay, hypotonia, and seizures. It is yet unclear whether these variants are causative and whether variants in CASP are associated with another distinct disorder. Therefore, we did not include these individuals in the phenotypic description of the present cohort. In addition, we gathered information on two neonates with a de novo heterozygous null variant in CUX1. However, for the phenotypic characterization, we only included postnatal individuals.

**CUX1 genotypic spectrum**

In 30 individuals, we identified a heterozygous CUX1 null variant: two splice-site variants, three gross deletions, one inversion, one translocation, eight nonsense, and 15 frameshift variants. Four individuals harbor heterozygous missense variants (Fig. 1). All variants were absent in gnomAD, except the variant c.2398delC. Of the 29 individuals for whom we were able to conduct a segregation analysis, 22 had de novo variants, while seven inherited the variant from a milder affected parent (Table S1). All but four truncating variants will likely result in nonsense-mediated mRNA decay (NMD). The variant c.61C > T will likely escape NMD as it creates a stop codon within the 3'UTR of exon 24. Although it might not cause impaired CUX1 activity.

Regarding the missense variants, three of these affect highly conserved amino acid residues. However, in silico predicting programs render only slightly increased scores. Furthermore, the de novo missense variant c.4064C > T (p.(Thr1355Ile)) affects a weakly conserved residue predicted to be benign by several in silico prediction programs (Table S2).

**Analysis of Cux1 heterozygous mice**

To investigate the underlying mechanisms of the Cux1 haploinsufficiency, we characterized a previously described mouse line that carries a truncating deletion in the homeodomain of Cux1. Although there were no previously reported alterations in the development of these heterozygous animals [13], we observed a slight reduction of early postnatal growth (Fig. 5A). Histological analysis of brain structures revealed no differences between Cux1+/− and WT animals (Fig. 5B).

We next quantified Cux1 transcripts in WT and Cux1+/− mouse cortices using RT-qPCR. This analysis evaluated the expression only of the WT isoforms and not the mutant transcripts (see Fig. 2A, B). As the clinical course of individuals suggests different impacts of heterozygosity at distinct developmental stages, we analyzed transcripts in the cortex of both young postnatal (P10) and mature (P30) animals. Cux1 transcripts were reduced to half the levels of WT, both in P10 and P30 Cux1+/− cortices (Fig. 2C).

To investigate CUX1 expression in the cortex of heterozygous mice, we immunostained brain coronal sections of P10 and P30 animals and quantified CUX1 levels in several cortical areas and layers, using an antibody against the C-terminal region of CUX1 but not CASP (see Fig. 3A). As expected, Cux1 null alleles affect exons not present in CASP, we found no changes in the expression of CASP transcripts (Fig. 2D). This indicates that the expression of the WT allele is not upregulated to compensate for the null allele in Cux1+/− mice.
As different CUX1 isoforms show different transcriptional activity [1, 4], we analyzed protein expression using western blot. Of note, the antibody used for immunofluorescence is unfortunately unsuitable for western blot. The available antibody for immunoblotting recognizes the common N-terminal region included in p200 CUX1 and CASP but not the shorter CUX1 isoforms. This analysis demonstrated a significant reduction in the expression of p200 CUX1 in Cux1+/− mice compared to WT at all tested ages (Fig. 3G, H). The blots also confirmed that CASP expression is unaffected in Cux1+/− mice (Fig. 3G). As control of antibody specificity, we confirmed the absence of the p200 CUX1 band in lysates from E18 Cux1−/− embryos. In both Cux1−/− and

**Fig. 2** The levels of WT Cux1 transcripts are reduced in the cortex of Cux1+/− mice. A Exon structure of Cux1 genomic sequence and detail showing the variant deleting exons 23 and 24 in Cux1+/−. Vertical lines represent individual exons. Arrows highlight primer sequences used for the quantifications of WT transcripts by RT-qPCR. B Predicted transcripts coding for CUX1 and CASP. Boxes and dashed boxes highlight the regions containing the RT-qPCR amplicons used to quantify protein-coding transcripts. The Cux1 amplicon measures all annotated CUX1 protein-coding transcripts (Cux1-201, 204, 209, 212, 206) except Cux1-208. RT-qPCR amplicon for CASP measures all annotated CASP protein-coding transcripts (Cux1-211, 207, 205). C Relative expression of Cux1 protein-coding transcript isoforms (Cux1-201, 204, 209, 212, 206) as shown in (A) and (B) at P10 and P30, quantified by RT-qPCR. Data are shown normalized to P10 WT levels. Data show mean ± SEM (n ≥ 3 animals per condition. Two-way ANOVA: P-value WT vs. Cux1+/− # # # # ≤ 0.0001. Post hoc with Tukey’s test: P-value P10 WT vs. Cux1+/− ** ≤ 0.005, P-value P30 WT vs. Cux1+/− ** ≤ 0.005). D Relative gene expression of protein-coding CASP transcripts at P10 and P30. Data are shown normalized to P10 WT levels. Data show mean ± SEM (n ≥ 3 animals per condition. Two-way ANOVA: P-value WT vs. Cux1+/− # ≤ 0.05. Post hoc with Tukey’s test: P-value P10 WT vs. Cux1+/− = 0.4641 (n.s.), P-value P30 WT vs. Cux1+/− = 0.1846 (n.s.).

As different CUX1 isoforms show different transcriptional activity [1, 4], we analyzed protein expression using western blot. Of note, the antibody used for immunofluorescence is unfortunately unsuitable for western blot. The available antibody for immunoblotting recognizes the common N-terminal region included in p200 CUX1 and CASP but not the shorter CUX1 isoforms. This analysis demonstrated a significant reduction in the expression of p200 CUX1 in Cux1+/− mice compared to WT at all tested ages (Fig. 3G, H). The blots also confirmed that CASP expression is unaffected in Cux1+/− mice (Fig. 3G). As control of antibody specificity, we confirmed the absence of the p200 CUX1 band in lysates from E18 Cux1−/− embryos. In both Cux1−/− and
Cux1<sup>−/−</sup> cortices, we also detected low levels of the mutant truncated CUX1 reported in previous studies [13] (Fig. 3G).

Thus, western blots showed equal reductions of p200 CUX1 expression at all ages. At the same time, immunofluorescence indicated a more significant deficiency of total CUX1 during development than in adulthood. These observations suggest that the lower immunofluorescence reductions in adults are likely due to immunoreactivity from short CUX1 isoforms. As P10 and P30 Cux1<sup>−/−</sup> mice show decreases in all transcripts, it is conceivable that post-transcriptional mechanisms balance the amount of...
Fig. 3  Cux1 cortical expression is reduced in heterozygous mice. A Comparative scheme of distinct functional areas in human (top) and mouse (bottom) brains. Top, dorsal (left), and lateral (right) views of motor (MO, green), somatosensory (SS, blue), auditory (AUD, magenta), and temporal association (TeA) cortical areas in the human brain. Bottom, dorsal (left), and medio-lateral views at several anteroposterior coordinates (right) of the functional areas in the mouse brain. B Intensity maps of Cux1 expression early in development (P10) in WT and Cux1+/− mice brains. Images show coronal sections from more anterior (left) to more posterior (right) coordinates. Dashed boxes in the middle images highlight areas of interest (SSbf, somatosensory barrel field). Scale bar = 500 µm. C, E Magnified images of cortical upper-layer neurons (L2-4) from dashed areas of Fig. S1B at P10 (C) and P30 (E). Scale bar = 200 µm. D, F Quantification of Cux1 expression in upper-layer neurons of SS, SSbf, and TeA areas at P10 (D) and P30 (F). Data show mean ± SEM (n ≥ 3 animals per condition). P10 Two-way ANOVA: *P-value WT vs. Cux1+/− ≤ 0.0001. Post hoc with Sidak’s test: **P-value SSbfL4 WT vs. Cux1+/− ≤ 0.01, **P-value SSbfL4 WT vs. Cux1+/− ≤ 0.01, P-value telescopic WT vs. Cux1+/− ≤ 0.01, P-value TeA WT vs. Cux1+/− ≤ 0.01. P30 Two-way ANOVA: *P-value WT vs. Cux1+/− ≤ 0.0001. G Western blot showing cortical expression of the full-length 200 kDa CUX1 in WT, Cux1+/−, and Cux1−/− (E18 only) mice at E18, P10, P30, and P135. The amount of protein was quantified and normalized to α tubulin expression (50 kDa). CASP (75 kDa), an alternatively spliced product of the Cux1 gene, is also recognized by this antibody. The truncated mutant CUX1 is indicated by an arrowhead. H Relative cortical expression of the 200 kDa CUX1 at P10, P30, and P135. Data show mean ± SEM. (n=3–4 cortical samples per condition. P10 unpaired t test: P-value WT vs. Cux1+/− ≤ 0.005. P30 unpaired t test: P-value WT vs. Cux1+/− ≤ 0.001. P135 unpaired t test: P-value WT vs. Cux1+/− ≤ 0.001).

Fig. 4  Cux1 heterozygosity results in increased seizure susceptibility in mice. A Identification of stages of seizure in a kainate-induced model of epilepsy. Photographs show representative behaviors from the less severe to the most, progressing from non-convulsive to convulsive seizure stages (R1-R6). B The maximum stage of seizure was reached in WT and Cux1−/− mice during the first 2 h of kainate induction. Data show mean ± SEM (n = 10 per condition. Mann–Whitney test: P-value WT vs. Cux1−/− ≤ 0.01). C Latency to the onset of convulsive stages (R3) in WT and Cux1−/− mice after kainate induction. Data show mean ± SEM (n ≥ 8 per condition. Mann–Whitney test: P-value WT vs. Cux1−/− = 0.3478 (n.s.)).

shorter CUX1 isoforms in older animals by proteolysis of p200 CUX1.

Finally, as eight individuals in the cohort had seizures, we analyzed the susceptibility of Cux1−/− mice to seizures upon administration of kainic acid (Fig. 4A). Only Cux1−/− and not WT animals developed severe attacks (Fig. 4B). There was, however, no difference in latency to the intermediate epileptic stage R3 (Fig. 4C). Thus, correlating with clinical findings, Cux1−/− mice demonstrated increased epileptic susceptibility compared to WT.

DISCUSSION

In the present study, we describe 34 individuals with CUX1 variants showing haploinsufficiency and refine the phenotypic spectrum of the CUX1-related neurodevelopmental disorder (NDD), including 11 previously published individuals [14–16]. Disease-causing variants in CUX1 lead to a non-specific phenotype with mild to moderate developmental delay/ID with possible developmental catch-up. 31 and 24 out of 32 assessable individuals presented with speech and motor delay, respectively. Among the individuals for whom information was available (n = 27), 21 exhibited a variable degree of ID, and 13 out of 31 and 8 out of 32 assessable individuals presented with hypotonia or seizures, respectively. Intrafamilial variations were observed in three out of six families (individuals 6, 7, 18, 19, 20 and 22 were comparable affected; Table S1). Generally, in these families, a null variant was transmitted from mildly affected parents to children with mild to moderate ID. Given that null variants follow a comparable pathomechanism, we also observed intrafamilial variations. In 23 families with null variants, ranged the degree of cognitive impairment from learning difficulties to moderate ID (Table 1, and Table S1). We observed additional non-neurological symptoms, such as joint laxity and short stature (Table 1). We were unable to identify any recurring specific facial dysmorphic features.
but the facial analysis showed a significant similarity between individuals 6, 7, 25 and 28 (Fig. S3). Although speculative, it is possible that a combination of multiple minor facial features resulted in a match of these individuals. This question can only be answered by analysing a large number of images of affected individuals using approaches such as GestaltMatcher [24]. Furthermore, our results with the mouse model demonstrate a reduction of Cux1/CUX1 expression in heterozygotes and support a causative link between disease-causing CUX1 variants and epilepsy.

The present study confirms that null variants in CUX1 are a cause for NDD. Moreover, we also provide evidence for missense variants to be causative, as we described four individuals with de novo missense variants in CUX1 (Fig. 1). CUX1 is intolerant to missense variation, as significantly fewer germline missense variants of CUX1 are detected in healthy controls in gnomAD than expected (z-score for missense variants = 3.75 [22]). Remarkably, the missense variant (p.Arg265Ser) of individual 5 has also been reported as de novo in an individual with NDD (DDD study [30]), but we could not obtain a detailed clinical description of this individual. Structural modeling of the missense variants was not possible due to the lack of an available protein model fitting the CUX1 isoform (encoded by NM_001202543.2). CUX1 exhibits high sequence conservation with CUX2, another member of the same family of homeodomain transcription factors. Like CUX1, CUX2 is highly expressed in the cerebral cortex, and both are involved in the dendritic development of L2/3 neurons [9, 31]. A recurrent de novo missense variant within the first CUT repeat of CUX2 causes CUX2-related epileptic encephalopathy [32]. Recent reports found other CUX2 missense variants as genetic contributors to kainic acid-induced epilepsy [33]. Although the number of individuals with missense variants is very low, it is striking that all of them had seizures (Table 1). Moreover, our mouse studies suggested that epilepsy is part of CUX1-related NDD, thus supporting the notion that missense variants in CUX1 could indeed be causative. Nevertheless, further studies are needed to clarify whether missense variants cause CUX1-related NDD.

CUX1 encodes two alternative splicing proteins, CASP and CUX1. In contrast to CUX1, CASP contains no DNA binding domains and is presumed to be involved in the transport of Golgi enzymes [34]. In the present cohort, individuals 1–9 and 29–32 harbor variants that affected shared CASP and CUX1 exons, while the variants of individuals 10–28 and 33 involved only exons encoding for CUX1 (Fig. 1). Interestingly, the proportion of variants affected by motor developmental delay was higher in those who harbored a CUX1 variant that affected both proteins (12/13, 92%) compared to individuals with a variant that affected only CUX1 (14/20, 70%). Therefore, it is both possible that CASP contributes to the regulation of neuronal development or disruption of CASP-specific exons alters the expression of CUX1 [35]. Noteworthy, there is an increased density of truncating variants in the general population (gnomAD) in the CASP-specific C-terminal region (Fig. 1). As we collected individuals with a variant that affects only CASP and observed a phenotype different from the present cohort, future studies are needed to clarify the involvement of CASP variants.

Although most individuals in the current study had mild to moderate developmental delay/ID, we also identified two neonates with a severe phenotype. Individuals F1 and F2 died shortly after birth in the context of status epilepticus and refractory respiratory disorder, respectively. In both individuals, we found a de novo frameshift variant in CUX1 (Table S1). Based on the phenotypic-genotypic spectrum described in this study, it remains uncertain whether these de novo variants alone are causative. However, as Cux1+/− mice died shortly after birth, we cannot exclude a second undetected variant in trans or a hypomorphic allele in these individuals, resulting in a complete loss of CUX1 function.

Haploinsufficiency appears in genes that cannot fulfill their function with half the dose and whose expression cannot be homeostatically regulated by increasing transcription or reducing protein elimination [36]. On the other hand, in some cases, mutant proteins can act as dominant-negative factors in heterozygous and produce adverse phenotypes [37]. Overall, our data in Cux1+/− mice supports that the deficit in CUX1 proteins is a primary cause of cognitive disabilities in affected individuals. However, we found low but detectable levels of the truncated protein in heterozygous mice (Fig. 3G). Therefore, we cannot rule out that mutant proteins contribute to abnormal phenotypes in mice and humans.

Noteworthy, the reduction in the p200 CUX1 isoform is higher than the 50% decrease expected in a heterozygous. Mechanistically, perhaps this reduction results from abnormal activation of the proteolytic cleavage of p200 described in proliferating cells [1], to compensate for the loss of shorter isoforms. On the other hand, the deficits in CUX1 expression in young Cux1+/−/L4 neurons, which upregulate CUX1 expression upon sensory experience [25, 38], and the epileptic phenotype in adults, suggest that heterozygosis compromises the upregulation of CUX1 that takes place during activity-dependent responses. However, confirming this hypothesis requires further investigation and needs to be addressed in an independent study.

Finally, another notable observation is the minor differences in total CUX1 expression between P30 Cux1−/− and WT mice compared to the differences in P10 animals. This observation opens possible speculations on the disease mechanism, considering the developmental catch-up observed in seven individuals. We do not have a record of disease trajectory of these individuals, but their cognitive functions were less affected than the others without catch-up (Table S1). Although one would expect null variants in CUX1 to be more common in the general population due to a variable expressivity or developmental catch-up, respectively, none of the variants occur in gnomAD, except for variant c.2393del (p.(Gln800Argfs*19)). To our knowledge, such a developmental catch-up is rarely seen in NDD individuals, but as genetic testing increasingly includes individuals with mild symptoms, such phenotypes may become more evident.

The limitations of this study are the unavailability of comprehensive phenotypic information for each individual, including IQ scores and clinical follow-up information.

In conclusion, we describe 34 individuals with potential causative variants in CUX1 and delineate the underlying neuro-developmental disorder. Most individuals had developmental delay and ID, and some of them confirmed the previously reported unusual developmental delay. Hypotonia and, to a lesser extent, seizures are part of the phenotypic spectrum. While truncating variants made up the bulk of underlying causal variants, our data suggest that rare de novo missense variants could also lead to CUX1-related NDD. Furthermore, our studies in mice indicate that CUX1-related NDD is due to insufficient production of CUX1 transcripts and that at the protein level, neurons of mature brains partly compensate for this reduction, which could sustain the possible amelioration of IQ reduction in affected human adults.

**DATA AVAILABILITY**

All data concerning this work is included in the manuscript and its supplement. Genetic variants reported in this study have been submitted to ClinVar or DECIPHER and they can be accessed using the URL https://www.ncbi.nlm.nih.gov/clinvar/ (Individual ID: SCV001335372.1; SCV001431662.1; SCV002764608; SCV002764609; SCV002764610; SCV002764611; SCV002764612; SCV002764613; SCV002764614; SCV001572223.1; SCV001335373.1; SCV001335398.1; SCV002764615; SCV002764616; SCV001335391.1; SCV002764617; SCV002764618; SCV002764619; SCV001335392.1; SCV002764620; SCV002764621; SCV002764622; SCV002764623; SCV001523945.1) and https://www.deciphergenomics.org/ (Individual ID: 401777; 387322; 503181; 338131; 322029; 503182; 503183; 503185; 503186).
ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41431-023-01445-2.

Correspondence and requests for materials should be addressed to Henry Oppermann, Marta Nieto or Zeynep Türner.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2023