De novo and biallelic *DEAF1* variants cause a phenotypic spectrum

Maria J. Nabais Sá, MD, PhD¹, Philip J. Jensik, PhD², Stacey R. McGee, MS², Michael J. Parker, MD³, Nayana Lahiri, MD⁴, Evan P. McNeil, MS⁵, Hester Y. Kroes, MD, PhD⁶, Randi J. Hagerman, MD^{7,8}, Rachel E. Harrison, MD⁹, Tara Montgomery, MD¹⁰, Miranda Splitt, MD¹⁰, Elizabeth E. Palmer, MBBS, BA^{11,12}, Rani K. Sachdev, MD^{11,12}, Heather C. Mefford, MD, PhD¹³, Abbey A. Scott, BS, MS¹⁴, Julian A. Martinez-Agosto, MD^{15,16}, Rüdiger Lorenz, MD¹⁷, Naama Orenstein, MD^{18,19}, Jonathan N. Berg, MD^{20,21}, Jeanne Amiel, MD²², Delphine Heron, MD²³, Boris Keren, MD, PhD²³, Jan-Maarten Cobben, MD^{24,25}, Leonie A. Menke, MD, PhD²⁴, Elysa J. Marco, MD²⁶, John M. Graham Jr, MD, ScD²⁷, Tyler Mark Pierson, MD, PhD²⁸, Ehsan Ghayoor Karimiani, MD, PhD²⁹, Reza Maroofian, PhD²⁹, M. Chiara Manzini, PhD³⁰, Edmund S. Cauley, MSc³⁰, Roberto Colombo, MD^{31,32}, Sylvie Odent, MD³³, Christele Dubourg, PharmD, PhD³⁴, Chanika Phornphutkul, MD³⁵, Arjan P. M. de Brouwer, PhD¹, Bert B. A. de Vries, MD, PhD¹ and Anneke T. Vulto-vanSilfhout, MD, PhD¹

Purpose: To investigate the effect of different *DEAF1* variants on the phenotype of patients with autosomal dominant and recessive inheritance patterns and on *DEAF1* activity in vitro.

Methods: We assembled a cohort of 23 patients with de novo and biallelic *DEAF1* variants, described the genotype–phenotype correlation, and investigated the differential effect of de novo and recessive variants on transcription assays using *DEAF1* and *Eif4g3* promoter luciferase constructs.

Results: The proportion of the most prevalent phenotypic features, including intellectual disability, speech delay, motor delay, autism, sleep disturbances, and a high pain threshold, were not significantly different in patients with biallelic and pathogenic de novo *DEAF1* variants. However, microcephaly was exclusively observed in patients with recessive variants ($p < 0.0001$).

Conclusion: We propose that different variants in the *DEAF1* gene

result in a phenotypic spectrum centered around neurodevelopmental delay. While a pathogenic de novo dominant variant would also incapacitate the product of the wild-type allele and result in a dominant-negative effect, a combination of two recessive variants would result in a partial loss of function. Because the clinical picture can be nonspecific, detailed phenotype information, segregation, and functional analysis are fundamental to determine the pathogenicity of novel variants and to improve the care of these patients.

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INTRODUCTION

DEAF1 (MIM *602635; NM_021008.3) encodes the deformed epidermal autoregulatory factor-1 homolog (DEAF1), a transcription factor that is highly expressed in the central nervous system, in particular during early embryogenesis.¹ *DEAF1* regulates the expression of various genes² as both a transcriptional activator and repressor.^{3–6} It contains several functional domains including a centrally located SAND (Sp-100, AIRE, NucP41/75, and DEAF1) domain, a zinc finger motif (ZnF), a nuclear localization signal (NLS), a nuclear

export signal (NES), and an MYND (myeloid translocation protein 8, Nery, and DEAF1) domain (Fig. 1) (refs. ^{6,7}). The region encompassing the SAND domain constitutes a DNA-binding domain, which binds to TTCG motifs.⁶ The SAND and MYND domains are also involved in protein–protein interactions.^{6–9} The presence of a NLS and NES indicates that DEAF-1 may be regulated by nuclear/cytoplasmic shuttling.⁷ Biallelic disruption of *Deaf1* in mice results in neural tube defects,¹⁰ and biallelic *Drosophila Deaf1* loss-of-function mutants show early embryonic arrest.¹¹

Correspondence: Bert Vries (Bert.deVries@radboudumc.nl). *Affiliations are listed at the end of the paper.

These authors contributed equally: Maria J. Nabais Sá, Philip J. Jensik

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Pathogenic variants in the *DEAF1* gene have been reported to lead to two clinically distinct intellectual disability (ID) syndromes: autosomal dominant mental retardation 24 (MRD24; MIM 615828) caused by de novo variants,^{1,12–17} and the recessively inherited dyskinesia, seizures, and

intellectual developmental disorder syndrome (DYSEIDD; MIM 617171) (refs. ^{16,18–20}). These two syndromes are collectively described as *DEAF1*-associated neurodevelopmental disorders (DAND). To date, nine different de novo pathogenic variants have been described in ten individuals,

Fig. 1 Genotype and facial phenotype of individuals with *DEAF1*-associated neurodevelopmental disorders (DAND). **a** Structure of the *DEAF1* precursor messenger RNA (pre-mRNA) [NM_021008.3] with light blue boxes indicating the 5' and 3' untranslated regions (UTRs) and dark blue boxes representing the coding region of exons 1 to 12, connected by lines indicating introns (not drawn to scale). **b** Structure of *DEAF1* protein [NP_066288], including the DNA-binding and dimerization SAND domain, the zinc finger homology domain (ZnF) also involved in DNA-binding, nuclear localization signal (NLS), nuclear export signal (NES), and MYND domain. Distribution of **(c)** de novo heterozygous variants leading to autosomal dominant mental retardation 24 and **(d)** homozygous or compound heterozygous variants leading to autosomal recessive dyskinesia, seizures, and intellectual developmental disorder. The number of patients described with the depicted variant are indicated between parentheses if the variant was identified in more than one case. Novel variants are indicated in bold. **e** Nonspecific facial dysmorphisms of individuals with pathogenic de novo *DEAF1* variants (**A–L**) and biallelic *DEAF1* variants (**M–P**), showing common characteristics as horizontal eyebrow, broad nasal tip, exaggerated Cupid's bow, thick lower lip vermillion, and pointed chin: (**A**) individual with p.(Gly212Ser), at 20 years old (AD/1); (**B**) individual with p.(Thr213Pro), at 7 years old (AD/3); (**C**) individual p.(Leu214Val), at 14 years old (AD/4); (**D**) individual with p.(Leu214Pro), at 3 years old (AD/5); (**E**) individual with p.(Lys216Glu), at 10 years old (AD/6); (**F**) individual with p.(Lys216Asn), at 7 years old (AD/7); (**G**) individual with p.(Pro174_Gly222del), at 6 years old (AD/8); (**H**) individual with p.(Gly225Glu), at 5 years old (AD/9); (**I**) individual with p.(Ser236Gly), at 4 years old (AD/11); (**J**) individual with p.(Arg254del), at 25 years old (AD/13); (**K**) individual with p.(Leu272Ser), at 2 years old (AD/15); (**L**) individual with p.(Ala276Pro), at 14 years old (AD/17); (**M**) individual with p.(Asp369Alafs*51) and p.(Cys540Metfs*18), at 17 years old (AR/1); (**N**) individual with p.(Trp234*) and p.(Glu239Gly), at 16 years old (AR/3); (**O**) individual with p.(Arg224Gln) in homozygosity, at 10 years old (AR/4); (**P**) individual with p.(Arg224Gln) in homozygosity, at 2 years old (AR/5). Individuals with de novo variants may have high forehead and/or macrocephaly, while individuals with biallelic variants may have narrow forehead and microcephaly.

who manifested moderate to severe ID with severely affected expressive speech and mild motor delay.^{1,12–17} Epilepsy was described in half of the individuals.¹⁶ Behavioral problems consisted of hyperactive, compulsive, and/or aggressive behavior; fascination with water; and striking mood swings.^{1,14–16} Other characteristics were autism, recurrent infections, a high pain threshold, and an abnormal walking pattern.^{1,14–16} Body measurements were normal, facial dysmorphisms were only mild, and no other major congenital anomalies were observed.¹ All but one of the de novo pathogenic *DEAF1* variants occurred in the SAND domain, including seven missense variants and one splice-site variant.^{1,12–17} One in-frame deletion of three base pairs was reported in the NLS domain.¹⁶ These de novo pathogenic *DEAF1* variants impair the *DEAF1* transcriptional activity, DNA-binding, and/or alter subcellular localization.^{1,16} Because heterozygous *DEAF1* deletions²¹ do not cause DAND, de novo pathogenic variants have been proposed to lead to a dominant-negative effect.¹

Two kindreds with patients with homozygous DYSEIDD pathogenic *DEAF1* variants were previously reported: a missense change and a noncanonical splice-site pathogenic variant.^{16,18–20} In total, seven patients homozygous for either of these variants presenting with ID, microcephaly, and hypotonia, were described.^{16,18–20} Seizures and white matter abnormalities resulting in dyskinesia were reported in four of the six patients for whom these features were investigated.^{18–20} The missense variant was present in the SAND domain as well, whereas the splice-site variant resulted in exon skipping and reduced the normal full-length messenger RNA (mRNA) copy number in the patients to 5% of the wild-type (WT) level,¹⁹ suggesting that the recessive phenotype resulted from a partial loss of function of *DEAF1*.

To investigate the effect of different *DEAF1* variants on the phenotype of patients with autosomal dominant and recessive inheritance patterns, we have assembled a cohort of 23 patients not previously reported with de novo and biallelic *DEAF1* variants. We describe the

genotype–phenotype correlation and investigate the differential effect of de novo and recessive variants on *DEAF1* activity in vitro.

MATERIALS AND METHODS

Identification of individuals

Patients were referred by physicians working in intellectual disability support services and by clinical geneticists. Written informed consent was obtained for all individuals involved, including consent to publish patient photos. This study adhered to the World Health Association Declaration of Helsinki (2013) and was approved by the institutional review board Commissie Mensgebonden Onderzoek Regio Arnhem-Nijmegen. Genomic DNA was isolated from peripheral blood samples following standard procedures.²² Exome enrichment, high-throughput sequencing, and subsequent de novo analysis was performed by established procedures.²³ Copy-number variant analysis was performed on exome data by using CoNIFER^{1,24} as described previously.²⁵

Plasmid constructs

DEAF1 mammalian expression and *DEAF1* and *Eif4g3* promoter luciferase plasmids have been previously described.¹ Site-directed mutagenesis was used to introduce the specific amino acid substitutions into the *DEAF1* expression plasmids, derived from the human *DEAF1* complementary DNA (cDNA) (GenBank accession number AF049459). Variants resulting in the indicated human amino acid substitutions were generated by polymerase chain reaction (PCR) as previously described.¹

Transcription assays

Luciferase assays using *DEAF1* and *Eif4g3* promoter luciferase constructs have been previously described.¹ Briefly, HEK293T cells in 24-well plates were transfected with 125 ng pcDNA3 (control) or *DEAF1* (WT or *DEAF1* variants) expression plasmids with 375 ng promoter luciferase and 1.25 ng RSV-Renilla luciferase constructs using the calcium phosphate technique for 18 hours. Luciferase assays were

performed 24 hours later using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

DEAF1 EBV-LCL RNA analysis

Epstein–Barr virus–transformed lymphoblastoid cell line (EBV-LCL) cells (8×10^6 cells) were grown in 20 mL Roswell Park Memorial Institute medium (RPMI) supplemented with 15% fetal bovine serum, penicillin, and streptomycin; 24 hours later, 10 mL was removed to a new flask and cells were treated with vehicle (water) or cycloheximide (50 μ g/mL) for 6 hours. RNA was isolated from EBV-LCL cells using Trizol reagent and 1.0 μ g of RNA was reverse transcribed. PCR was performed using cDNA and primers to amplify specific regions of DEAF1 cDNA for downstream restriction digests or subcloning and DNA sequencing. Quantitative PCR was performed using DEAF1 (1617dup) and GAPDH primers²⁶ and data were normalized using the $2^{(-\Delta\Delta Ct)}$ method.²⁷

Immunoprecipitation and western blot

EBV-LCLs were lysed in lysis buffer containing 150 mM sodium chloride, 50 mM Tris (pH = 7.5), 1.0% TritonX-100, 1.0 mM EDTA, 1.0 mM sodium fluoride, 0.2 mM sodium orthovanadate, aprotinin (10 μ g/mL), leupeptin (10 μ g/mL), and pepstatin (10 μ g/mL) and lysates were incubated with rabbit DEAF1 antibodies⁸ or preimmune serum bound to protein G magnetic beads overnight at 4°C. Beads were washed and proteins were eluted in Laemmli sample buffer. Transfected HEK293t cells were lysed in lysis buffer. Proteins were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to polyvinylidene fluoride membranes. Immunoblot analysis was performed with a different rabbit anti-DEAF1 antibody for EBV-LCL immunoprecipitation experiments or rabbit anti-DEAF1 and mouse anti-BACTIN (Abcam, Cambridge, MA, USA) for transfected HEK293t experiments.

Genotype–phenotype correlations

Fisher's exact test, using a two-sided *p* value, was carried out to compare proportions of clinical features between groups of patients with de novo and biallelic *DEAF1* variants. After conducting Bonferroni correction, a *p* value of <0.0025 was considered significant.

RESULTS

De novo variants in the SAND domain of DEAF1 impair transcriptional activity

We identified 14 novel and two previously published de novo *DEAF1* variants in a total of 18 patients by exome sequencing (*n* = 16), genome sequencing (*n* = 1), and autism gene panel analysis (*n* = 1) performed in 17 medical centers from all over the world (Fig. 1; Table S1). These variants included 14 missense variants, one in-frame deletion that resulted in a single amino acid change, and one splice-site variant, c.664 +1G>T. To determine if the c.664 +1G>T variant resulted in alternatively spliced DEAF1 mRNA transcripts, PCR was used to amplify regions containing exons 2–5 using EBV-LCL

mRNA from individual AD/8. DNA sequencing indicated the variant causes skipping of exon 4, which results in an in-frame deletion of 49 amino acid residues in the SAND domain p.(Pro174_Gly222del) (Fig. 2). Compared with control EBV-LCL, a truncated DEAF1 protein, as well as full-length DEAF1, were observed in patient AD/8 EBV-LCL lysate and corresponded to a similar molecular weight protein using lysates from cells transfected with WT or p.(Pro174_Gly222del) plasmid DNA. The novel variant p.(Ala276Pro) was found in two unrelated individuals from this study. The previously reported de novo variants p.(Gly212Ser) and p.(Gln264Pro)^{1,13,16} were identified in our cohort in two and one families, respectively. The p.(Gly212Ser) variant was identified in 2 of 247 reads in peripheral blood cells of the patient's unaffected father. The variant was not detected by Sanger sequencing, suggesting that he is likely a mosaic for this variant.

Fifteen of the 16 de novo variants are located in the SAND or adjacent ZnF domain; the p.(Pro293Leu) variant is between the ZnF and NLS (Fig. 1c). All missense variants were predicted by SIFT²⁸ and PolyPhen-2 (ref. ²⁹) to be disease-causing and had a CADD score >20.0 (ref. ³⁰). None of the novel de novo variants were found in gnomAD.³¹ Multi-species alignment showed that all mutated amino acids are highly conserved across species (Figure S1).

DEAF1 binds and represses its own promoter activity.³² *DEAF1* promoter transcriptional repression activity was analyzed for de novo variants. All variants lost transcriptional repression activity compared with wild-type DEAF1, although p.(Pro293Leu) retained some repression activity (Fig. 3a). DEAF1 also increases the transcriptional activity of the mouse *Eif4g3* promoter⁵ and de novo *DEAF1* variants have previously been shown to suppress activation of this promoter.¹ In 12 of our 14 variants, an approximate tenfold suppression of transcriptional activity relative to basal expression levels was demonstrated. No activation or reduction in promoter activity was observed for p.(Ser236Gly) relative to basal activity, and p.(Pro293Leu) increased promoter activity (Fig. 3b).

Biallelic variants in DEAF1 result in no significant effect in transcriptional assays

Six novel biallelic *DEAF1* variants were found in compound heterozygosity in three unrelated individuals and one novel homozygous variant was identified in two siblings of a consanguineous family by using exome sequencing (Fig. 1d; Table S2). These variants were scattered throughout *DEAF1*. In two families, compound heterozygous variants, p.(Trp234*) and p.(Glu239Gly), or a homozygous variant, p.(Arg224Gln), were found in the SAND domain. The compound heterozygous missense variant p.(Glu239Gly) is not reported in gnomAD and is predicted by SIFT to be deleterious, by PolyPhen-2 to be probably damaging, and had a CADD score 25. The homozygous substitution p.(Arg224Gln) located in the SAND domain is present only once heterozygously in gnomAD (frequency of 4.065e-6), was

Fig. 2 The de novo variant c.664+1G>T results in an in-frame deletion within the *DEAF1* SAND domain. **a** Location of the c.664+1G>T variant relative to exons 2–5 in the *DEAF1* gene. **b** Polymerase chain reaction (PCR) using primers that would amplify exons 2–5 of *DEAF1* messenger RNA (mRNA) (gray bars shown in **a**) with complementary DNA (cDNA) from control or c.664+1G>T Epstein–Barr virus–transformed lymphoblastoid cell line (EBV-LCL) (individual AD/8) treated with vehicle (–) or cycloheximide (CHX) (+). A single DNA band was observed in control EBV-LCL corresponding to correctly spliced *DEAF1*. An additional, approximately 150-bp smaller PCR product was identified in c.664+1G>T EBV-LCL. **c** DNA sequencing indicated c.664+1G>T EBV-LCL has both correctly spliced *DEAF1* RNA (above) as well as *DEAF1* mRNA that lacks exon 4 (below), which could result in a truncated p.Pro174_Gly222del *DEAF1* protein. **d** Lysates from HEK293T cells transfected with the expression constructs for wild-type (WT) (~80 kDa) or p.Pro174_Gly222del variant (~75 kDa) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by western blot using DEAF1 (red) and b-ACTIN (green) antibodies. **e** Lysates from control or c.664+1G>T EBV-LCL were immunoprecipitated with preimmune serum (PI) or DEAF1 antibodies followed by western blots with DEAF1 antibodies. The control EBV-LCL shows a single 80-kDa full-length DEAF1 protein band. An additional ~75 kDa DEAF1 protein was also observed in the EBV-LCL with the c.664+1G>T variant. Closed arrows indicate DEAF1 protein bands and open arrow indicates heavy chain IgG (IgG HC).

predicted by SIFT to be tolerated, by PolyPhen-2 to be disease-causing, and had a CADD score 31. In the other two families, a single base-pair duplication or deletion resulting in frameshift near to the 5' of *DEAF1*, p.(Arg44Glyfs*25), or in the middle of the *DEAF1* gene, p.(Asp369Alafs*51), both located outside a domain, occurred in combination with a variant in the MYND domain, respectively an in-frame deletion, p.(Phe527del) or an insertion resulting in a frameshift, p.(Cys540Metfs*18). Three of these variants, p.(Arg44-Glyfs*25), p.(Trp234*), and p.(Asp369Alafs*51), are expected to lead to nonsense-mediated decay (NMD) of their respective transcripts.

DEAF1 mRNA expression analysis was performed using EBV-LCL from patient AR/1, who carried c.1617dup, p.(Cys540Metfs*18) and c.1104_1105dup, p.(Asp369Alafs*51) in compound heterozygosity, and from parents carrying either the c.1617dup or c.1104_1105dup. To differentiate the expression of *DEAF1* transcripts from each allele, two different PCR amplicons were generated that contain either c.1617dup or c.1104_1105dup variant from patient and

parent EBV-LCL treated with vehicle or cycloheximide (CHX), an inhibitor of NMD. The c.1617dup eliminates an endogenous *NdeI* site and the c.1104_1105dup generates a new *HhaI* site. Restriction endonuclease digestion analysis indicated that both c.1617dup or c.1104_1105dup containing mRNA transcripts were present in patient and parent cDNA. The c.1104_1105dup transcript seemed to be present in a lower amount in cells not treated with CHX as compared with those treated with CHX. As CHX is an inhibitor of NMD, this suggests that this transcript is subject to NMD (Figure S2). *DEAF1* immunoprecipitations were performed on EBV-LCL lysates from patient and parents. Compared with parent EBV-LCL, no full-length or p.(Cys540Metfs*18) ~80-kDa *DEAF1* protein was observed in patient lysate. A faint truncated ~60-kDa band consistent with the p.(Asp369Alafs*51) *DEAF1* protein was observed in patient and parent c.1104_1105dup. Quantitative PCR (qPCR) showed that *DEAF1* mRNA expression in untreated EBV-LCL of the patient AR/1 (c.1104_1105dup/c.1617dup variants) was 30% of normal EBV-LCL expression levels ($p < 0.05$) (Figure S3). CHX

Fig. 3 Effect of *DEAF1* variants on transcriptional activity. **a, b** *DEAF1* promoter activity after transfection of HEK293t cells with wild-type (WT) or the indicated variants. **a** Compared with WT, all de novo variants resulted in loss of transcriptional repression activity at the *DEAF1* promoter. **b** Heritable biallelic variants had no effect on *DEAF1* promoter transcriptional repression, except for p.(Trp234*). **c, d** *Eif4g3* promoter activity after transfection of HEK293t cells with WT or the indicated variants. **(c)** All de novo variants, except p.(Pro293Leu) and p.(Ser236Gly), resulted in suppression of transcription relative to WT transactivation. No change from basal promoter activity (pcDNA3) was detected for p.(Ser236Gly) and increased promoter activity was observed for p.(Pro293Leu). **d** Heritable biallelic variants had no effect on *Eif4g3* promoter activation compared with WT. Each bar represents the mean $\pm$ SEM of the normalized luciferase activity of three independent experiments when the activity of pcDNA3 (*DEAF1* promoter alone) was set to 100% ($N = 5-7$ de novo variants with $N = 14-15$ WT, $N = 4-5$ heritable variants with $N = 10-12$ WT). ** $p < 0.01$ One-way analysis of variance (ANOVA) with Dunnett's multiple comparison WT *DEAF1* versus each mutant.

treatment significantly increased *DEAF1* mRNA expression relative to vehicle-treated cells in the c.1104_1105dup/c.1617dup patient as well as the parent carrying the c.1104_1105dup supporting that the c.1104_1105dup-containing transcript is targeted by NMD.

The effects of the biallelic variants on *DEAF1* transcriptional activity, protein expression, and cellular localization were also determined. Compared with WT *DEAF1*, no change in transcriptional repression activity was observed for the biallelic variants, except for p.(Trp234*) (Fig. 3c). No significant change in transcriptional activation of the *Eif4g3* promoter was observed (Fig. 3d). Interestingly, p.(Arg44-Glyfs*25), which should result in a severely truncated *DEAF1* protein that lacks most of the *DEAF1* protein domains including the SAND domain, retained transcriptional activity. Western blots were performed using lysates from HEK293t transfected with WT or *DEAF1* biallelic

variants. A faint ~70-kDa protein was observed for p.(Arg44Glyfs*25) (Figure S4). This might be the result of the use of a translational start at a downstream in-frame AUG start codon located at codon 69. The p.(Arg44Glyfs*25) variant results in a stop codon 5' adjacent to methionine 69 of *DEAF1*. The p.(Trp234*) and p.(Asp369Alafs*51) variants resulted in truncated *DEAF1* proteins approximately 30 and 60 kDa in size, respectively. The p.(Cys540Metfs*18) variant resulted in a slightly lower molecular weight protein. As expected p.(Arg224Gln), p.(Glu239Gly), and p.(Phe527del) variants had the same apparent molecular weight as WT *DEAF1*. Immunofluorescent staining was used to determine the subcellular localization of the biallelic *DEAF1* variants in transfected cells. Unlike WT *DEAF1*, which localized to the nucleus, p.(Trp234*) was found throughout the cell (Figure S5). The other biallelic variants localized to the nucleus.

De novo *DEAF1* variants result in a nonspecific phenotype comprising ID, speech delay, motor delay, sleep disturbance, autism, and a high pain threshold

Fourteen male and three female individuals with a de novo *DEAF1* variant (median age: 7.5 years; age range: 2.5–38 years), were clinically characterized (Table 1; Table S3). All were born from healthy unrelated nonconsanguineous families. This cohort consisted of 11 children, 6 of whom were preschool children, 3 were adolescents, and 3 were adults. One individual was excluded from genotype–phenotype analysis because he had the variant p.(Pro293Leu), which was not located in the SAND domain and did not result in suppression, but rather in an increase of Eif4g3 promoter activity.

Developmental delay (DD)/ID was present in all patients (17/17; 100%) and was typically moderate–severe or severe DD/ID (Table S4). Motor delay, present in most of the patients (12/17; 71%), was usually mild. Language development was severely delayed in all patients, except for one patient with the variant p.(Leu214Val) (16/17; 94%). Seven patients had absent speech and nine had limited speech. Of note, nonverbal patients were said to have used a few words before losing the ability to produce speech. Developmental regression was described in half of the individuals, mostly corresponding to loss of the ability to communicate verbally. Although in most individuals expressive language was severely impaired, receptive language was perceived, particularly by caregivers, as better than expressive. Furthermore, patients (15/15; 100%) were able to communicate simple needs nonverbally, for instance taking the caregivers' hand to point or lead to the item(s) of interest, using gestures and sign language, producing sounds accompanied (or not) by gestures (e.g., to express emotions, as annoyance), or using a picture exchange communication system, picture prompts, a tablet, or a touch-based speech-generating device.

Autism spectrum disorder occurred in all patients (16/16; 100%). In the majority, poor eye contact (12/16; 75%); fascinations (15/17; 88%), particularly a compulsive interest in water; and sudden mood swings (16/17; 94%) were concomitantly observed. Mood swings were characterized by exaggeration of emotional affects, with paroxysmal bursts of laughter and context-inappropriate happy disposition alternating with aggressive behavior or depressive feelings. Aggressive behavior (12/16; 75%) manifested both as heteroaggression and autoaggression (e.g., self-mutilation, head-banging, self-biting). Other behavioral problems (13/15; 87%) included pica, impulsivity/hyperactive behavior, and obsessive traits.

Epilepsy was also observed in the majority of patients (10/14; 71%). Median age at diagnosis was 3 years, although age of onset varied from 2 days old to 16 years old (Table S5). Seizures were usually generalized and frequently-difficult to treat or intractable (9/10; 90%). Antiepileptic drugs (AEDs) were able to reduce seizures in five patients, while in four other patients seizures were refractory to

treatment. Of note, seizures were noticed to adversely influence speech.

Sleep dysfunction was frequent and severe (15/17; 88%). It consisted of difficulty falling asleep (sleep onset insomnia) and awaking frequently (maintenance insomnia). Patients were reported to be very disruptive when waking during the night. In some patients, sleeping problems were treated with some benefit using medication, such as melatonin.

Other neurologic abnormalities included a remarkably high pain threshold (14/16; 88%), hypotonia (10/16; 63%), and gait difficulties, comprising of gait ataxia (4/16; 25%) or an ataxic looking gait (12/17; 71%), as broad-based and imbalanced gait, or a tip-toe gait. Brain abnormalities as indicated by brain magnetic resonance image (MRI) were present in less than a third of the patients (4/13; 31%), but no common pattern of brain malformations could be distinguished. Movement disorder, in particular dystonia, was only reported in one individual.

Non-neurodevelopmental issues included recurrent infections that occurred in more than half of patients (10/16; 63%), mostly in infancy or childhood. They included predominantly chronic ear infections, sinusitis, or upper respiratory tract infections. In addition, gastrointestinal abnormalities were common (12/16; 75%) and included feeding difficulties, gastroesophageal reflux disease, constipation, and diarrhea. Craniofacial dysmorphisms were mild (Fig. 1e) and macrocephaly was observed in approximately one quarter of individuals (4/17; 24%). Growth was usually within the norm for age and gender.

Biallelic *DEAF1* variants result in microcephaly

Microcephaly (3/5; 60%) was exclusively observed in patients with recessive variants. All patients with biallelic *DEAF1* variants manifested ID and developmental regression, except for one (Tables S6 and S7). All parents were healthy. Remarkably, although the patient with variants p.(Asp369Alafs*51) and p.(Cys540Metfs*18) in compound heterozygosity never learned to walk and had absent speech, similarly to other patients with biallelic *DEAF1* variants, he completed various exams from the first grade of secondary school, suggesting he has a normal cognitive function, although this was not formally tested. Additionally, he used nonverbal communication aids (supportive typing) that revealed good receptive language. Behavioral problems occurred in all patients and ranged from poor eye contact (4/5; 80%) to autism (1/4; 25%) and aggressive behavior (2/5; 40%). Frequent neurologic abnormalities included epilepsy (4/5; 80%) (Tables S5), sleep disturbance (5/5; 100%), and brain abnormalities (4/5; 80%), such as ventriculomegaly with or without hydrocephalus, corpus callosum hypoplasia, or white matter abnormalities. Movement disorder (2/4; 50%) included dystonia and stereotypies. Common extraneurologic features included feeding difficulties (3/5; 60%) and constipation (5/5; 100%). Lastly, facial dysmorphisms were minor and non-specific (Fig. 1e).

Table 1 Phenotype of patients with pathogenic de novo and biallelic variants in the *DEAF1* gene

	Patients with de novo <i>DEAF1</i> variants		Patients with biallelic <i>DEAF1</i> variants			p value ^c
	This study (N = 17)	Published ^a (N = 10)	Total (N = 27)	This study (N = 5)	Published ^b (N = 7)	Total (N = 12)
Gender (female/male)	3 F:14 M	6 F:4 M	9 F:18 M	2 F:3 M	1 F:6 M	3 F:9 M
Median age (range)	7.5 years (2.5–38 years)	10 years (3–15 years)	9.5 years (2.5–38 years)	9 years (2–17 years)	4 years (2–20 years)	8.5 years (2–20 years)
Growth						
Microcephaly	0/17	0/4	0/21 (0%)	3/5	3/3	6/8 (75.0%)
Macrocephaly	4/17	0/4	4/21 (19.0%)	1/5	0/3	1/8 (12.5%)
Psychomotor development						
DD/ID	17/17	9/9	26/26 (100%)	4/5	7/7	11/12 (91.7%)
Developmental regression	8/16	3/5	11/21 (52.4%)	4/5	0/1	4/6 (66.7%)
Behavioral abnormalities						
Poor eye contact	12/16	3/4	15/20 (75.0%)	4/5	NA	4/5 (80.0%)
Autism	16/16	8/9	24/25 (96.0%)	1/4	3/3	4/7 (57.1%)
Mood swings	16/17	3/4	19/21 (90.5%)	3/5	3/3	6/8 (75.0%)
Fascinations	15/17	2/2	17/19 (89.5%)	3/5	NA	3/5 (60.0%)
Aggressive behavior	12/16	2/2	14/18 (77.8%)	2/5	1/1	3/6 (50.0%)
Other behavioral problems	13/15	1/1	14/16 (87.5%)	2/5	1/1	3/6 (50.0%)
Neurologic abnormalities						
Seizures	10/14	5/5	15/19 (78.9%)	4/5	4/6	8/11 (72.7%)
Hypotonia	10/16	4/7	14/23 (60.9%)	3/5	4/6	7/11 (63.6%)
Gait ataxia	4/16	2/2	6/18 (33.3%)	1/2	NA	1/2 (50.0%)
Other walking problems ^d	12/17	4/4	16/21 (76.2%)	1/2	NA	1/2 (50.0%)
High pain threshold	14/16	4/4	18/20 (90.0%)	1/3	NA	1/3 (33.3%)
Sleep disturbance	15/17	4/6	19/23 (82.6%)	5/5	3/3	8/8 (100%)
Brain MRI abnormalities	4/13	2/4	6/17 (35.3%)	4/5	5/6	9/11 (81.8%)
Other abnormalities						
Feeding difficulties	9/16	1/5	10/21 (47.6%)	3/5	2/2	5/7 (71.4%)
Constipation	7/16	1/5	8/21 (38.1%)	5/5	NA	5/5 (100%)
Recurrent infections	10/16	4/4	14/20 (70.0%)	2/5	0/1	2/6 (33.3%)

DD developmental delay, ID intellectual disability, MRI magnetic resonance image.
^aVisser et al.¹² (2010), Rauch et al.¹³ (2012), Vulto et al.¹ (2014), Wenger et al.¹⁴ (2017), Berger et al.¹⁵ (2017), Chen et al.¹⁶ (2017), Shi-Jun et al.¹⁷ (2018).
^bFaqeih et al.¹⁸ (2014), Rajab et al.¹⁹ (2015), Gund et al.²⁰ (2016), Chen et al.¹⁶ (2017).
^cA p value <0.0025 was considered significant, after conducting a Bonferroni correction.
^dOther walking problems were described as tip-toe gait (n = 4), broad-based gait (n = 4), gait imbalance (n = 3), clumsiness (n = 2), unsteady gait (n = 1), waddling gait (n = 1), walk with feet rotated laterally (n = 2), and slow walking (n = 1). Some individuals displayed more than one of these features.

De novo and biallelic variants result in a similar phenotype except for microcephaly

The proportion of the most prevalent phenotypic features, including ID, speech delay, motor delay, autism, sleep disturbances, and a high pain threshold, was not significantly different in patients with biallelic and pathogenic de novo *DEAF1* variants, when considering all patients included in this study and previously described individuals (Table 1). Inter-familial phenotypic variability was observed among patients with the same de novo variant (e.g., some patients developed seizures, while others did not). Microcephaly, however, was observed exclusively in patients with recessive variants ($p < 0.0001$).

DISCUSSION

In this study, we expanded the clinical consequences of variants implicated in *DEAF1*-associated neurodevelopmental disorders (DAND) by describing the phenotype of 17 patients with a de novo pathogenic variant and 5 patients with biallelic variants in the *DEAF1* gene. We determined that the de novo variants impaired *DEAF1* transcriptional repression activity and 15 of the 17 variants reversed *DEAF1*-mediated transcriptional activation at the *Eif4g3* promoter. Apart from the p.(Trp234*) variant, the other six novel recessive *DEAF1* variants demonstrated no change in transcriptional activity.

We identified missense variants in the SAND domain, both de novo and recessive. Only de novo variants, however, altered *DEAF1* and *Eif4g3* promoter activity. This is in line with previous data implying a dominant-negative effect of these variants.¹ So far, no de novo nonsense or frameshift variants resulting in disease have been identified. De novo variants clustered in very close proximity within the SAND domain. Moreover, variants have been found recurrently affecting the same residues, namely p.Gly212, p.Leu214, p.Lys216, p.Ile228, p.Arg254, p.Gln264, and p.Ala276 (refs. ^{1,12–20}). Additionally, the canonical donor splice site 3' of c.664 was also found to be repeatedly affected.¹⁷ We show in our study that c.664+1G>T causes skipping of exon 4, producing an in-frame deletion in the SAND domain that results in abnormal transcriptional activity mimicking the effect of other missense variants on *DEAF1* protein activity.

Missense biallelic variants in the SAND domain do not impair the function of this domain as evidenced by the normal transcriptional activity of the mutant protein in our assays. This is especially remarkable for the novel homozygous variant p.(Arg224Gln), as the previously reported de novo p.(Arg224Trp) variant was shown to result in loss of transcriptional repression of the *DEAF1* promoter, and was unable to activate the *Eif4g3* promoter.¹ We hypothesize that the p.(Arg224Gln) variant affects normal protein folding of *DEAF1* because arginine, an amino acid with an electrically charged side chain, is replaced by an amino acid with a polar uncharged chain. This is also the case for the recessive p.(Arg226Trp) variant, also located in the SAND domain.^{16,18,20} Similarly, the compound heterozygous variant p.(Glu239Gly), which results in a protein with an apparently normal length,

may have also an effect on the normal protein folding, due to the substitution of the electrically charged side chain of a glutamate for a glycine with a small nonpolar side chain. It is possible the *DEAF1* variants could influence other aspects of *DEAF1* function or activity that may not be detected by the cell-based functional assays used in this study. Thus, we predict that these variants constitute a classical, but incomplete, loss of function (hypomorph).

Loss-of-function recessive variants in *DEAF1* consist of p.(Arg44Glyfs*25), p.(Trp234*), p.(Asp369Alafs*51), and p.(Cys540Metfs*18). Only p.(Trp234*) affected transcriptional repression activity. This variant likely results in a truncated protein lacking most of the SAND domain. Our results suggest that the *DEAF1* transcript containing the p.(Arg44-Glyfs*25) variant can be transcribed using an alternative start codon resulting in a N-terminal deletion of 69 amino acid residues, and thus in an intact SAND domain. Accordingly, these data indicate the MYND domain is not essential for *DEAF1* transcriptional repression or activation activity using the in vitro assays in this study. Taken together, the de novo SAND domain variants likely have a dominant-negative effect (antimorph), whereas the biallelic *DEAF1* variants lead to a partial loss of function (hypomorph) through either a reduction in function with normal protein levels (missense variants) or reduced protein levels due to NMD (nonsense and frameshift variants). This hypothesis also explains why heterozygous deletions of *DEAF1* do not result in a phenotype.

The phenotype did not differ significantly between patients with de novo and biallelic *DEAF1* variants, except for the presence of microcephaly ($p < 0.0001$). The latter feature was present in three of the five patients with biallelic variants. Patients with de novo or biallelic *DEAF1* variants had DD/ID with severe speech delay, autism, striking mood swings, epilepsy, gait ataxia or other walking problems, a high pain threshold, sleep disturbance, brain abnormalities, and extraneurologic manifestations, such as recurrent infections and gastrointestinal abnormalities that included feeding difficulties, constipation, and/or diarrhea. This suggests that DAND consists of a phenotypic spectrum of features centered around neurodevelopmental delay rather than two distinct clinical syndromes, but a larger number of patients will be required to confirm this hypothesis. To further delineate the clinical spectrum associated with de novo variants in *DEAF1*, we established a website to collect detailed clinical information of additional individuals to be identified over the coming years (<http://humandiseasesgenes.nl/deaf1/>). As such, the clinical picture may be quite nonspecific. Syndromes to be included in DAND differential diagnosis would be Angelman syndrome, mostly due to ID, gait abnormalities, seizures, paroxysmal bursts of laughter, and fascination with water; Smith-Magenis syndrome, in particular due to the combination of ID and severe sleeping problems;¹⁵ and Rett syndrome for the biallelic *DEAF1* variants, due to the combination of microcephaly and regression.

The exclusive occurrence of microcephaly and an increased prevalence of some phenotypic abnormalities, such as movement disorder and recurrent structural brain abnormalities, in patients with recessive *DEAF1* variants may also suggest that patients with recessive *DEAF1* variants may present with a different and somewhat more severe clinical condition than patients with de novo variants, as these variants have a different effect on a molecular level. One exception to the classical DAND phenotype was observed in one patient with biallelic *DEAF1* variants as he has preserved cognitive abilities. Nevertheless, this patient with variants p.(Asp369Alafs*51) and p.(Cys540Metfs*18) in compound heterozygosity shared clinical features with other patients with biallelic *DEAF1* variants, specifically inability to walk or to speak,^{16,18–20} movement disorder,¹⁹ and minor brain abnormalities including corpus callosum, ventricles, and white matter abnormalities.^{18,20} Another exception to the classical DAND phenotype is the occurrence of normal speech in one patient with the de novo dominant variant p.(Leu214Val), who manifests ID, showing the phenotypic spectrum is wider than previously described.

In conclusion, we propose that different variants in Figure S1 the *DEAF1* gene result in a phenotypic spectrum centered around neurodevelopmental delay. A pathogenic de novo dominant variant would incapacitate the product of the wild-type allele and result in dominant-negative effect, whereas a combination of two recessive variants would result in a partial loss of function. Because the clinical picture can be quite nonspecific, detailed phenotype information, segregation analysis, and functional analysis are fundamental to determine the pathogenicity of novel variants and to improve the care of these patients.

SUPPLEMENTARY INFORMATION

The online version of this article (<https://doi.org/10.1038/s41436-019-0473-6>) contains supplementary material, which is available to authorized users.

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DISCLOSURE

The authors declare no conflicts of interest.

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¹Department of Human Genetics, Radboud University Medical Center, Nijmegen, The Netherlands. ²Department of Physiology, Southern Illinois University School of Medicine, Carbondale, IL, USA. ³Sheffield Clinical Genetics Service, OPD2 Northern General Hospital, Sheffield, UK. ⁴Department of Clinical Genetics, St George’s University Hospitals NHS Foundation Trust & St George’s, University of London, London, UK. ⁵Dartmouth Geisel School of Medicine, Hanover, NH, USA. ⁶Department of Genetics, University Medical Center Utrecht, Utrecht, The Netherlands. ⁷Medical Investigation of Neurodevelopmental Disorders (MIND) Institute, University of California Davis School of Medicine, Sacramento, CA, USA. ⁸Department of Pediatrics, University of California Davis Medical Center, Sacramento, CA, USA. ⁹Department of Clinical Genetics, Nottingham University Hospitals NHS Trust, Nottingham, UK. ¹⁰Northern Genetics Service, Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle upon Tyne, UK. ¹¹Sydney Children’s Hospital, Randwick, NSW, Australia. ¹²School of Women’s and Children’s Health, UNSW Medicine, The University of New South Wales, Sydney, NSW, Australia. ¹³Department of Pediatrics, Division of Genetic Medicine, University of Washington–Seattle, Seattle, WA, USA. ¹⁴Division of Genetic Medicine, Seattle Children’s Hospital, Seattle, WA, USA. ¹⁵Department of Human Genetics, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA. ¹⁶Division of Medical Genetics, Department of Pediatrics, David Geffen School of Medicine at UCLA, Los Angeles, CA, USA. ¹⁷Ludwig-Konrad-Str. 14, Bad Wildungen, Germany. ¹⁸Pediatric Genetics Clinic, Schneider Children’s Medical Center of Israel, Petach Tikva, Israel. ¹⁹Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel. ²⁰Department of Clinical Genetics, Ninewells Hospital and Medical School, Dundee, Angus, UK. ²¹Clinical Genetics, University of Dundee, Dundee, Angus, UK. ²²Département de Génétique, Hôpital Necker-Enfants Malades, Assistance Publique, INSERM UMR 1163, Institut Imagine, Paris, France. ²³Département de Génétique, Hôpital Pitié-Salpêtrière, Assistance publique–Hôpitaux de Paris, Paris, France. ²⁴Department of Pediatrics, Amsterdam University Medical Centers, Amsterdam, The Netherlands. ²⁵North West Thames Genetics NHS, Northwick Park Hospital, London, UK. ²⁶Department of Child Neurology, Cortica Healthcare, San Rafael, CA, USA. ²⁷Division of Clinical Genetics and Dysmorphology, Department of Pediatrics, Cedars-Sinai Medical Center, Los Angeles, CA, USA. ²⁸Department of Pediatrics, Department of Neurology, and the Board of Governors Regenerative Medicine Institute, Cedars-Sinai Medical Center, Los Angeles, CA, USA. ²⁹Genetics Research Centre, Molecular and Clinical Sciences Institute, St George’s, University of London, London, UK. ³⁰GW Institute for Neuroscience, Department of Pharmacology and Physiology, The George Washington University School of Medicine and Health Sciences, Washington, DC, USA. ³¹Faculty of Medicine “Agostino Gemelli” Catholic University of the Sacred Heart, Rome, Italy. ³²Center for the Study of Rare Inherited Diseases (CeSMER), Niguarda Ca’ Granda Metropolitan Hospital, Milan, Italy. ³³Service de Génétique Clinique, CLAD-Ouest CHU Rennes, Univ Rennes, CNRS 6290 Institut de Génétique et Développement de Rennes (IGDR), Rennes, France. ³⁴Univ Rennes, CHU Rennes, CNRS, IGDR, UMR 6290, Rennes, France. ³⁵Division of Human Genetics, Department of Pediatrics, Hasbro Children’s Hospital, Warren Alpert Medical School of Brown University, Providence, RI, USA