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A novel UBE3A sequence variant identified in eight related individuals with neurodevelopmental delay, results in a phenotype which does not match the clinical criteria of Angelman syndrome

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Abstract

Background: Loss of functional UBE3A, an E3 protein ubiquitin ligase, causes Angelman syndrome (AS), a neurodevelopmental disorder characterized by severe developmental delay, speech impairment, epilepsy, movement or balance disorder, and a characteristic behavioral pattern. We identified a novel *UBE3A* sequence variant in a large family with eight affected individuals, who did not meet the clinical AS criteria.

Methods: Detailed clinical examination and genetic analysis was performed to establish the phenotypic diversity and the genetic cause. The function of the mutant UBE3A protein was assessed with respect to its subcellular localization, stability, and E3 ubiquitin ligase activity.

Results: All eight affected individuals showed the presence of a novel maternally inherited *UBE3A* sequence variant (NM_130838.4(UBE3A):c.1018-1020del, p.(Asn340del), which is in line with a genetic AS diagnosis. Although they presented with moderate to severe intellectual disability, the phenotype did not match the clinical criteria for AS. In line with this, functional analysis of the UBE3A p.Asn340del

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mutant protein revealed no major deficits in UBE3A protein localization, stability, or E3 ubiquitin ligase activity.

Conclusion: The p.(Asn340del) mutant protein behaves distinctly different from previously described AS-linked missense mutations in UBE3A, and causes a phenotype that is markedly different from AS. This study further extends the range of phenotypes that are associated with UBE3A loss, duplication, or mutation.

KEYWORDS

Angelman syndrome, E3 protein ubiquitin ligase, intellectual disability, missense variant, UBE3A

1 | BACKGROUND

Angelman syndrome (AS: OMIM #105830), first described in 1965 by Harry Angelman (Angelman, 1965), is a rare neurodevelopmental disorder with a prevalence of 1:20.000 (Bird, 2014; Larson, Shinnick, Shaaya, Thiele, & Thibert, 2015; Mertz et al., 2013; Prasad, Grocott, Parkin, Larson, & Thibert, 2018; Smith, 2001). It is caused by deficient expression or function of the maternally inherited *UBE3A* gene. The most common cause of AS is a maternal 15q11-q13 deletion. Other known genetic causes are paternal uniparental disomy, genomic imprinting defects, and intragenic variants in *UBE3A* (Buiting, Williams, & Horsthemke, 2016). Although these latter two groups tend to present with a milder phenotype, all these patients present with a well-defined AS phenotype, which is a profound to severe developmental delay, severe speech impairment, movement, or balance disorder such as ataxia of gait and/or tremulousness of the limbs, and a characteristic behavioral pattern. Other common features include epilepsy, microcephaly, and other characteristic facial features, sleep disturbance, scoliosis, strabismus, and constipation (Williams et al., 2006). The natural course of AS in adulthood has been less well described (Larson et al., 2015; Prasad et al., 2018; Smith, 2001).

The *UBE3A* gene (UBE3A; MIM #601623) encodes a HECT (homologous to the E6-AP carboxyl terminus) –type of E3 ubiquitin protein ligase. The C-terminal HECT domain harbors the catalytic cysteine residue that is essential for transferring the ubiquitin to either itself (auto-ubiquitination) or to substrate proteins, generally targeting the modified proteins for proteasomal degradation (de Bie & Ciechanover, 2011; Huibregtse, Scheffner, Beaudenon, & Howley, 1995). In human, three UBE3A protein isoforms are described that harbor different N-termini: the two long isoforms, isoforms 2 and 3, each have an N-terminal extension of, respectively, 23 and 20 amino acids compared to the short isoform (isoform 1) (Yamamoto, Huibregtse, & Howley, 1997). Mice express two protein isoforms, isoforms 2 and 3, which are highly homologous to the human isoforms 3 and 1, respectively (Avagliano Trezza et al., 2019; Greer et al., 2010). In human and mouse neurons UBE3A is mainly localized to the nucleus with smaller amounts present in axons and dendrites (Avagliano Trezza et al., 2019; Burette et al., 2016, 2018; Dindot, Antalffy, Bhattacharjee, & Beaudet, 2008). Recent work in mice has revealed how this subcellular distribution of UBE3A is achieved: the short isoform (mUBE3A Iso3), which constitutes ~80% of total UBE3A, is mainly nuclear while the, less abundant, long isoform (mUBE3AIso2) is predominantly present in the cytosol (Avagliano Trezza et al., 2019; Miao et al., 2013). Analysis of isoform-specific mice suggests that in vivo the nuclear isoform of UBE3A, but not the cytosolic isoform, has a critical role in normal neurodevelopment (Avagliano Trezza et al., 2019).

AS is often caused by a microdeletion of the *UBE3A* gene, but ~10% of the patients are reported to have pathogenic missense variants that change one or a few amino acids in the UBE3A protein (Avagliano Trezza et al., 2019; Baumer, Balmer, & Schinzel, 1999; Camprubí et al., 2009; Cooper, Hudson, Amos, Wagstaff, & Howley, 2004; Fang et al., 1999; Sadikovic et al., 2014; Yi et al., 2015). Understanding if, and how, these missense mutations contribute to the pathogenesis of AS remains a challenge. Specifically, it requires genetic and clinical information of the patient carrying the *UBE3A* variant as well as detailed functional analysis of the mutant protein. For most AS-linked missense mutations reported in the literature functional characterization of the mutant protein is lacking. Here we report the clinical phenotype and functional characterization of a novel maternally inherited *UBE3A* sequence variant (NM_130838.4(UBE3A):c.1018-1020del, p.(Asn340del) that was identified in a large family during a Dutch study on the natural course of AS in adulthood. The eight related individuals affected by this sequence variant do not, or only in a very limited manner, resemble AS and we provide a molecular explanation for the unusual mild phenotype of these patients.

2 | METHODS

2.1 | Ethical compliance

The study was approved by the Medical Ethical Review Board of the Erasmus Medical Center, Rotterdam (MEC-2015-267).

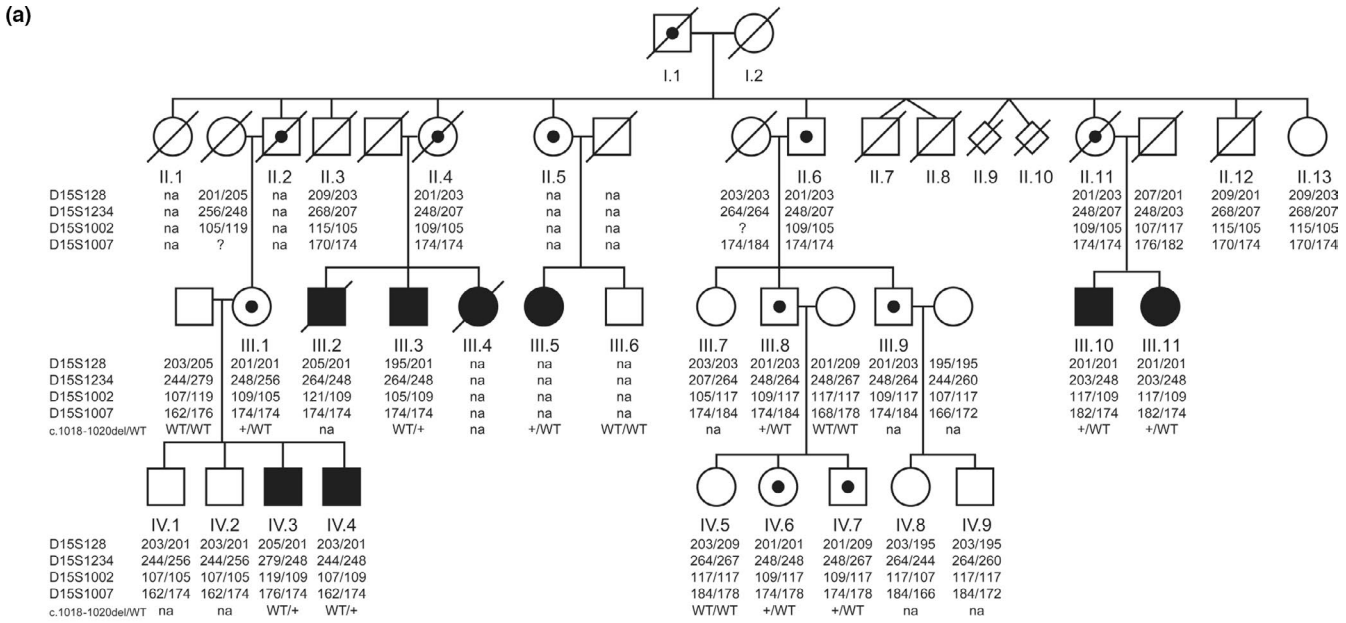

FIGURE 1 Pedigree of the family carrying hUBE3A-p.Asn340del variant. (a) Family tree including results of haplotyping and DNA studies. (b) Images of all living patients harboring the UBE3A p.Asn340del variant

Written informed consent was obtained from all individuals participating in the study.

2.2 | Recruitment of patients

From 2015 to 2016 adult individuals with genetically confirmed AS were included in a Dutch study to investigate the natural course of AS in adulthood. Parents or legal representatives were recruited for the study via the Dutch Angelman parents association (Vereniging Angelman Syndroom Nederland) and the Dutch Society of Physicians for persons with intellectual disabilities. Furthermore, adults visiting the outpatient clinic of the Dutch expertise Center ENCORE (Dutch Expertise Center which gathers expertise of rare congenital neuro-cognitive developmental disorders, such as AS) were approached to participate.

Parents or legal representatives who were interested in participating were sent information regarding the study to decide upon participation in the study. Written informed consent was thereafter obtained.

Individuals IV.3 and IV.4 (see Figure 1) were participants in the Dutch natural course study. Their mother informed us of other probands and approached her family members to participate. All alive probands in this family were visited and blood samples were collected to clarify the genetic origin of their cognitive impairment.

2.3 | Medical history and clinical examination

All participants were visited by the researchers (IdB, AGH and MJV). Medical history was obtained, medical records were studied, and a full clinical examination was performed.

Three questionnaires were filled in by parents or legal representatives. The first questionnaire was used to collect data about pregnancy, first clinical signs and symptoms, mental and motor milestones, behavioral problems, sleeping problems, epilepsy, hearing and vision, digestion, and medical history.

To further assess behavior and sleep another two questionnaires were used: the Aberrant Behavior Checklist (ABC) and Sleep Disturbance Scale for Children (SDSC) (Bruni et al., 1996; Marshburn & Aman, 1992). In addition, parents and/or caregivers were interviewed by Vineland Adaptive Behavior Scales (VABS) by one of the researchers. The VABS is a widely used instrument, which gives an indication of the developmental age (Sparrow & Cicchetti, 1985). The reliability of the Vineland in children and adolescents with mental retardation proved to be good and the construct validity is high (Aman, Singh, Stewart, & Field, 1985).

2.4 | Molecular analyses

Before inclusion in this study, patients had been studied at the Genome diagnostics laboratory of Radboudumc, Nijmegen, The Netherlands. In one individual (IV.4), at the age of 12 years, some features of AS were recognized, since he showed frequent laughter and clumsy movement, although not ataxia. The pattern of inheritance, in combination with the intellectual disability let to further suspicion of AS in these individuals. A deletion and paternal disomy at 15q11-13 were excluded, but linkage analysis using the following fluorochrome markers from proximal 15q11–q13: D15S128, D15S1234, D15S1002, and D15S1007, indeed assigned the causative genetic defect to 15q11-13 (for comparable analysis in another family see Meijers-Heijboer et al., 1992). Eventually the genetic defect underlying the AS was found in one of the individuals in 2002. In 2016 Individuals IV.3 and IV.4 (see Figure 1) applied for participation in the Dutch natural course study and their mother informed us of other probands and approached her family members to participate. All alive probands in this family were included and after inclusion in the study further genetic analysis was performed at the Erasmus MC, Rotterdam, The Netherlands. Genomic DNA was isolated from peripheral blood using standard procedures (Chemagen). Bidirectional sequencing of part of exon 7 of *UBE3A* was undertaken using forward primer (5′ TTGCAAAGCGATGAGCAAGCTACC 3′) and reverse primer (5′ TGGTCACGTCTAACTTTGAGTCTC 3′). PCR conditions are available upon request. Subsequently, the PCR products were purified and sequenced using BigDye Terminator chemistry v3.1 on an ABI Prism 3130xl genetic analyzer (Applied Biosystems). Sequences obtained were analyzed using Sequence Pilot software (JSI Medical Systems GmbH). The variant was annotation according to the Mutation Nomenclature guidelines from the Human Genome Variation Society (HGVS), using reference sequence RefSeq NM_130838.4. In silico splice site prediction was performed using Alamut software (Alamut Visual interactive biosoftware).

2.5 | Molecular biology

The construction of plasmids expressing mouse UBE3A isoform 3 (homologous to human *UBE3A* isoform 1) has been described previously (Avagliano Trezza et al., 2019). The p.Asn340del (p.Asn337del in mouse UBE3A) mutation was introduced in the wild-type UBE3A gene by site-directed mutagenesis (Forward: 5′ GAATTTAATAGCCGAAATCT AGTGGATGATGATGCCATTGTTG; Reverse: 5′ CAAC A A T G G C A T C A T C A T C C A C T A G A T T T C G GCTATTAAATTC). For the stability assay a plasmid

with an IRES2 (Internal Ribosomal Entry Site) element was constructed. First, the IRES2 fragment was amplified by PCR using pCAG-CRE-IRES2-GFP (Addgene) as a template in order to introduce a 5′ BamHI site and 3′ AgeI site. The resulting BamHI-AgeI fragment was inserted into pYE581. The multiple cloning site of the first open reading frame was replaced using a double-stranded oligonucleotide introducing an HA (hemagglutinin)-tag followed by the restriction sites AscI, PmeI, and NotI. mUBE3A-Iso3 (wild type and variants) was inserted AscI-NotI into the first open reading frame. The second open reading frame contains HA-GFP which was obtained by amplification of pEGFP-N3 (Clontech) in order to introduce a 5′ AgeI site followed by a HA-tag, and a 3′ BsrGI site. The resulting AgeI-HA-GFP-BsrGI fragment was used to replace the tdTomato in pYE581 using the AgeI site in the MCS and the internal BsrGI site of tdTomato. For bacterial ubiquitination experiments UBE3A constructs were cloned in a pRSF-Duet (Novagen)-derived vector. These UBE3A constructs harbor an HA epitope tag at their N-termini. Tagging at the N-terminus of UBE3A does not affect its ubiquitin ligase activity (Kühnle, Mothes, Matentzoglu, & Scheffner, 2013). The UBE3A target Ring1b-I53S (a catalytically inactive mutant of Ring1b, generous gift of Martin Scheffner) was cloned in pMB419, a derivative of pCOLA-Duet1 (Novagen). Details on the construction of the plasmids and validation of the bacterial ubiquitination system will be described elsewhere (R. Avagliano Trezza et al., manuscript in preparation).

2.6 | Bacterial ubiquitination assay

The bacterial ubiquitination assay can be used to recapitulate the mammalian ubiquitination cascade in *E. coli* cells (Keren-Kaplan et al., 2011). BL21-GOLD (DE3) cells were co-transformed with bacterial expression constructs encoding E1, UbcH5c, ubiquitin, as well as UBE3A variants, and the substrate RING1b-I53S. Transformants were selected on solid LB medium (1% (w/v) Bacto tryptone, 0.5% (w/v) Bacto yeast extract, 1% (w/v) NaCl, and 1.5% agar) with antibiotics as required and single colonies were inoculated in overnight culture of LB medium supplemented with 2% glucose and 50 mM Tris/HCl (pH 8.0). The following morning cells were inoculated at a starting OD_{600} of 0.2 and grown to OD_{600} 0.7 at 21°C. Upon reaching requisite OD_{600}, protein expression was induced by addition of 0.5 mM isopropyl B-D-1-thiogalactopyranoside (IPTG) and growth was continued at 16°C for 16–18 hrs. The following day, cells were lysed in 0.5 ml lysis buffer (50 mM sodium-phosphate pH 8.0, 300 mM NaCl, 5% glycerol, 5 mM 2-mercaptoethanol, 1 mM PMSF, RNase [0.01 mg/ml], DNase [0.01 mg/ml], and protease inhibitor cocktail) by sonication. Cellular debris was removed by centrifugation (30 min, 13,000× *g*) and cleared protein lysate was used for SDS-PAGE and western blotting.

2.7 | Stability in HEK293T cells

Protein stability of UBE3A variants was analyzed in HEK293T cells by transient transfection of mammalian expression constructs (2 μg DNA per sample) encoding UBE3A variants. Forty-eight hours post-transfection cells were harvested. Cell pellets were taken up in 1X Laemmli sample buffer (0.2 M Tris–HCl [pH 6.8], 1.5% sodium dodecyl sulfate [SDS], 10% glycerol, 1 mM EDTA, 0.004% bromophenol blue) containing protease inhibitor cocktail and cells were lysed by sonication (3 × 4 s, 5 mAmp). Protein lysate (10 μg) was analyzed by SDS-PAGE and western blotting.

2.8 | Cycloheximide chase

Protein stability of UBE3A variants was analyzed using a cycloheximide chase assay in HEK293T cells. HEK293T cells were transiently transfected with mammalian expression constructs (2 μg DNA per sample) encoding UBE3A variants. Twenty-four hours post-transfection cells were treated with cycloheximide [70 μg/ml) and chased across different time periods. At the conclusion of each time period cells were harvested in ice-cold PBS (Sigma) and briefly centrifugated (5 min, 1600× *g*). Cell pellets were taken up in 1X Laemmli sample buffer (0.2 M Tris–HCl [pH 6.8], 1.5% sodium dodecyl sulfate [SDS], 10% glycerol, 1 mM EDTA, and 0.004% bromophenol blue) containing protease inhibitor cocktail and cells were lysed by sonication (3 × 4 s, 5 mAmp). Protein lysate (15 μg) was analyzed by SDS-PAGE and western blotting.

2.9 | Western blot analysis

For western blot analysis, approximately 20 μg of total protein lysate per sample was separated by SDS-PAGE and transferred onto nitrocellulose membranes. Next, membranes were blocked in TBS (10 mM Tris–HCl (pH 8.0), 150 mM NaCl) containing 5% (w/v) powdered milk for 1 hr at room temperature, washed three times in TBS-T (TBS with 0.1% Tween-20, Sigma P1379) and incubated at 4 °C overnight, rotating end-over-end, with the primary antibody dissolved in TBS-T with 2% (w/v) milk solution. The day after, membranes were washed three times for 10 min with TBS-T and incubated with the secondary antibody dissolved in TBS-T with 2% (w/v) milk for 1 hr. At the end of the incubation, membranes were washed three times for 10 min with TBS and analyzed by measuring enhanced chemiluminescence (ECL).

Antibodies used for western blotting were: Mouse monoclonal anti-UBE3A (Sigma SAB1404508), Mouse monoclonal anti-V5 horseradish peroxidase (HRP)-conjugated (Thermo Fisher Scientific R961-25), Rat monoclonal anti-HA HRP-conjugated (Roche 12013819001), Mouse monoclonal anti-UBE3A (Sigma E8655), and Mouse monoclonal anti-GFP (Roche 11814460001).

3 | RESULTS

The pedigree of the family with the novel UBE3A variant is shown in Figure 1a, with images of all living patients in Figure 1b. Main clinical information is provided in Table 1, together with an overview of level of functioning regarding communication, activities of daily living and social and motor skills as given by the VABS in Table 2. Overall, the phenotype in probands in this family did not meet the clinical criteria of AS described in the Updated Consensus for Diagnostic Criteria of AS 2005 (Williams et al., 2006). Probands showed a milder degree of intellectual disability and were able to speak a few words or small sentences with the exception of individual III.2 who was not able to speak (Table 1). Affected individuals could walk long distances with only mild or no ataxia (Ind. III.3 and III.5). None of the affected individuals showed scoliosis or microcephaly. No hearing or visual deficits were observed except for individual IV.3 who showed mildly impaired vision and hearing. Facial features typical for AS such as protruding tongue, wide mouth, and widespread teeth were absent in

TABLE 1 Clinical features present in the probands

	III.2	III.3	III.4	III.5	III.10	III.11	IV.3	IV.4
Sex	M	M	F	F	M	F	M	M
Birth weight (grams)	NA	NA	NA	NA	NA	NA	3140	3220
Perinatal problems	NA	NA	NA	NA	−	NA	−	−
Age of walk (years)	NA	NA	NA	NA	NA	NA	3	3
Ataxia of gait	−	±	−	±	−	−	−	−
Tremors	−	±	−	+	−	−	−	±
Frequent laughter	−	−	−		−	−	−	+
Hyperactive behavior	+	−	−	−	−	−	−	−
Attention deficit	+	−	−	−	−	−	−	−
Hand flapping	−	−	−	−	−	−	−	−
Development delay	+	+	+	+	+	+	+	+
Speech	Absent	Sentences	Words	Words	Sentences	Sentences	Sentences	Sentences
Microcephaly	NA	−	NA	−	−	−	−	−
Seizures	−	−	−	+	−	+	−	−
Hypotonia	−	−	−	−	−	−	−	−
Feeding problems	−	−	−	+[a]	−	+[b]	+[c]	+[d]
Flat occiput	NA	−	NA	−	−	−	−	+
Hypopigmented skin	NA	−	NA	−	−	−	−	−
Strabismus	NA	−	NA	−	−	−	+	−
Wide mouth	NA	−	NA	−	−	−	−	−
Wide-spaded teeth	NA	−	NA	−	−	−	−	−
Protruding tongue	Na	−	NA	−	−	−	−	−
Scoliosis	−	−	−	−	−	−	−	−

Note: Clinical features as defined by Williams et al. (2006).

Abbreviations: NA, not available; −, absent; ±, mild; +, present.

[a]Swallowing problems.

[b]History of obesity.

[c]Obesity.

[d]Feeding problems as an infant, reflux.

TABLE 2 Vineland results

	III.3	III.5	III.10	III.11	IV.3	IV.4
Communication	23[a]	14	22	33	27	22
Activities of daily living	35	22	76	70	46	44
Social skills	21	8	33	39	42	57
Motor skills	36	16	56	56	34	41

[a]Developmental age in months per category.

the probands. Other clinical symptoms such a characteristic behavioral symptoms, epilepsy, and sleep disturbance were absent in most of the affected individuals. Hyperactive behavior and attention deficit was only observed in individual III.2 (male, †66 years). Probands III.3, III.10, IV.3, and IV.4 were socially outgoing and happy, enjoying the visits of the researchers, while III.11 was a bit reluctant. Since individuals with a comparable intellectual disability are frequently socially outgoing as well, the researchers find it difficult to indicate if this behavior is consistent with AS or appropriate for their developmental age. Sleep disturbance and epilepsy was seen in individuals III.5 and III. 11. Ind. III.5 (female, 57 years) had sleeping problems her whole life and got her first epileptic seizure at the age of 16. The total amount of epileptic seizures in her life is 10. For individual III. 11 (female, 43 years) no sleeping problems were reported, but she had 2–4 epileptic seizures during her life. Feeding problems, another typical phenotype of AS patients, were present in only four of the eight affected individuals (III.5, III.11, IV.3, and IV.4). Two affected individuals were found to have obesity due to a lack of satiety (III.11 and IV.3).

Sequencing analyses of *UBE3A* revealed the sequence variant NM_130838.4(UBE3A):c.1018-1020del, p.(Asn340del) on the maternally inherited allele in III.3, III.5, III.10, III.11, IV.3, and IV.4. Sequence analyses in III.6 revealed no nucleotide changes in UBE3A. In III.1 p.(Asn340del) was detected on the paternally inherited UBE3A copy. The variant leads to an in-frame deletion in a non-repetitive region. There was no predicted effect on splicing (Splice Site Finder Like, MaxEntScan, NNSPLICE, GeneSplicer). The sequence variant was not present in the public database gnomAD (V2.1).

Previous reports have shown that AS-linked variants in *UBE3A* may affect the function of UBE3A by changing its subcellular localization, E3 ubiquitin ligase activity, or stability (Avagliano Trezza et al., 2019; Cooper et al., 2004; Yi et al., 2015). The p.Asn340del variant is located in the N-terminal half of UBE3A well outside the catalytic HECT domain (Figure 2a). To determine if the p.Asn340del variant affects UBE3A localization we deleted the Asn residue of mouse UBE3A at position of 337, which is the homologous position in mouse UBE3A isoform 3, fused at its C-terminus to GFP (mUBE3A-iso3$^{N337\Delta}$-GFP). Mouse primary neurons transfected with mUBE3A-iso3$^{N337\Delta}$-GFP showed a predominant nuclear localization that was indistinguishable from that of wild-type mUBE3A-iso3-GFP, indicating that the p.Asn340Δ variant does not affect the localization of UBE3A (Figure 2b).To assess E3 ubiquitin ligase activity, we modified a previously established bacterial ubiquitination system where the mammalian ubiquitination cascade can be recapitulated by co-transforming bacterial expression constructs containing the requisite enzymes (Avagliano Trezza & Distel, unpublished data). We first tested the ability of mUBE3A-iso3$^{N337\Delta}$ to ubiquitinate itself, an activity also known as auto- of self-ubiquitination (Nuber, Schwarz, & Scheffner, 1998). In the absence of a target, the mUBE3A-iso3$^{N337\Delta}$ variant showed auto-ubiquitination levels comparable to wild-type mUBE3A (Figures 2c,e and S1). In the presence of target RING1b^{I53S} (a catalytic inactive variant unable to ubiquitinate itself) both wild-type mUBE3A and the mUBE3A-iso3$^{N337\Delta}$ variant show comparable levels of RING1B ubiquitination (Figures 2d,f and S1).

To determine if the mUBE3A-iso3$^{N337\Delta}$ variant affects protein levels in vivo we introduced the mutation in mUBE3A-iso3 and transfected the expression construct into HEK293T cells. Protein levels of the mUBE3A-iso3$^{N337\Delta}$ variant were compared to wild-type mUBE3A-iso3 and mUBE3A-iso3^{C21Y}, a variant that causes AS and has been previously shown to be unstable (Cooper et al., 2004; Yi et al., 2015). Quantification of the western blots revealed similar protein levels of mUBE3A-iso3$^{N337\Delta}$ compared to wild-type mUBE3A-iso3 ($p = 0.1331$), whereas the mUBE3A-iso3^{C21Y} variant was detected at significantly lower levels ($p = 0.0011$) (Figures 2g and S2). Next, we determined the protein stability of these UBE3A variants in a cycloheximide chase experiment (Figure 2h). This experiment showed that the stability of mUBE3A-iso3$^{N337\Delta}$, within the analyzed timespan, is similar to that of wild-type mUBE3A, whereas the mUBE3A-iso3^{C21Y} variant is already present at much lower levels and disappears within 8 hrs. Together these data show that nuclear localization, ubiquitin ligase activity, and stability of the mUBE3A-iso3$^{N337\Delta}$ protein are not significantly affected.

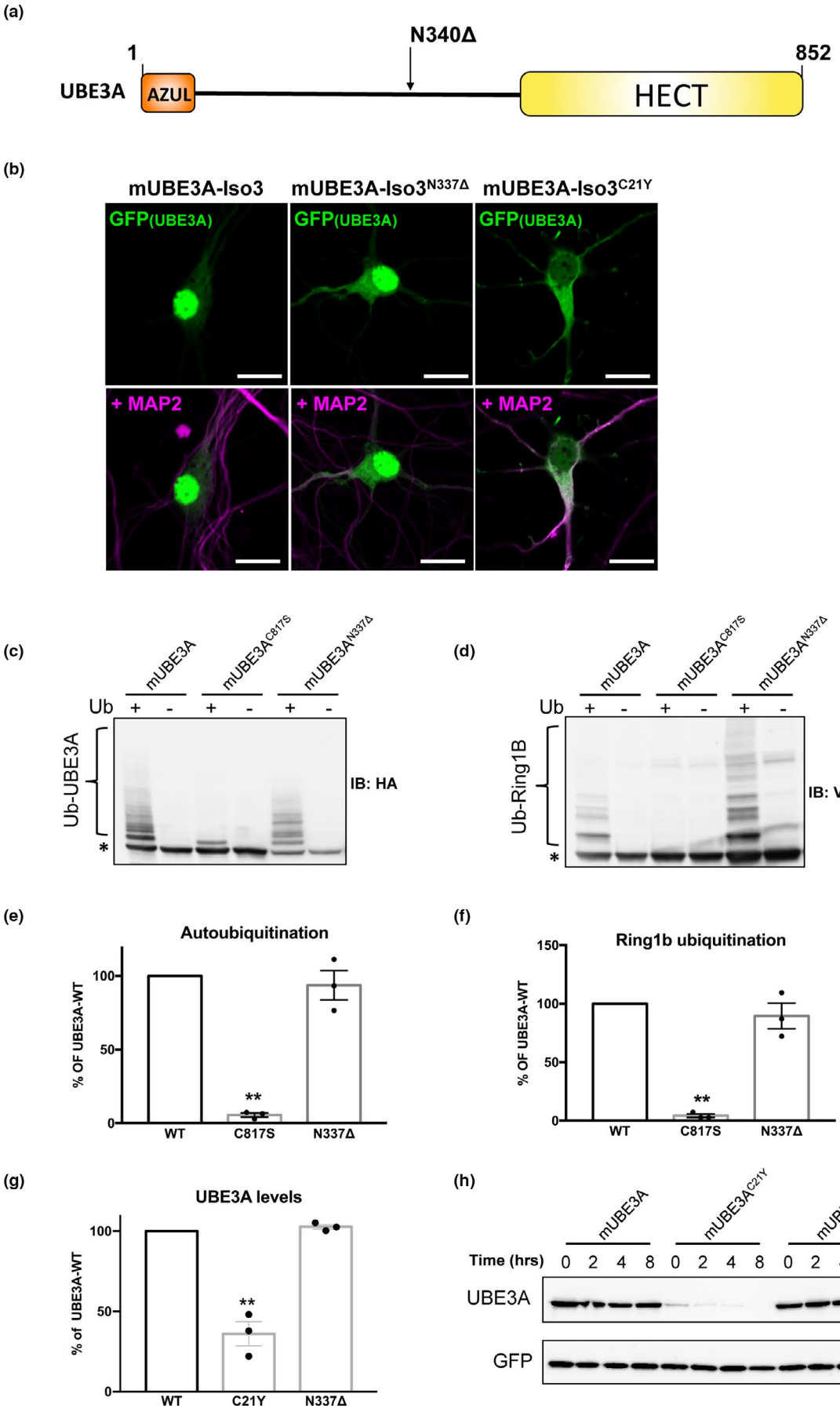
(a)
1
N340Δ
852
UBE3A
AZUL
HECT
(b)
mUBE3A-Iso3
mUBE3A-Iso3N337Δ
mUBE3A-Iso3C21Y
GFP(UBE3A)
+ MAP2
(c)
mUBE3A
mUBE3AC817S
mUBE3AN337Δ
Ub
Ub-UBE3A
IB: HA
(d)
Ub-Ring1B
IB: V5
(e)
Autoubiquitination
% OF UBE3A-WT
WT
C817S
N337Δ
(f)
Ring1b ubiquitination
(g)
UBE3A levels
% of UBE3A-WT
C21Y
(h)
Time (hrs)
0 2 4 8
UBE3A
GFP

FIGURE 2 Functional analysis of the mUBE3A-p.Asn337del variant (homologous to hUBE3A-p.Asn340del). (a) Schematic representation of human UBE3A isoform 1 with functional domains, N-terminal Zn-binding domain (AZUL) and C-terminal HECT (homologous to E6AP C-terminus) domain, and location of the p.Asn340del mutation. (b) Localization of mUBE3A-Iso3 and the p.Asn337del variant in neurons. E16.5-derived hippocampal neurons transfected at 4 days in vitro (DIV4) with the indicated mUBE3A-Iso3 variants C-terminally tagged with GFP, were fixed and stained at DIV8. UBE3A-GFP expression was detected by direct fluorescence (green, top panels) together with MAP2 (pink, lower panels). *E. coli* cells expressing E1, UbcH5 (E2) and with or without ubiquitin were transfected with a plasmid expressing mouse HA-tagged mUBE3A (wild-type, catalytically inactive [C817S] or Asn337del) (c) or with the same HA-mUBE3A variants and V5-RING1B-I53S, a catalytically inactive RING1B variant that is unable to ubiquitinate itself (d). Cell lysates were analyzed by SDS-PAGE and immunoblotting using HA antibody to visualize UBE3A (c) and V5 antibody to visualize RING1B (d). (e, f) Quantification of catalytic activity. Values are shown as mean percentage ± SD of WT UBE3A activity (set at 100%). $N = 3$, $*p < 0.05$, $**p < 0.01$. (g) Quantification of expression levels of mUBE3A constructs. HEK293T cells were transiently transfected with mUBE3A, mUBE3A$^{N337\Delta}$ or mUBE3A^{C21Y} and protein levels were determined by anti-UBE3A western blotting. All data represent mean ± SD and are normalized against actin. Wild-type (WT) UBE3A levels were set to 100%. $N = 3$, $**p < 0.05$. (h) Protein stability of mUBE3A variants. HEK293T cells transfected with the indicated mUBE3A expressing plasmids and a plasmid expressing GFP (transfection control). Twenty-four hours post-transfection, cells were treated with cycloheximide (70 μg/ml) and chased across different time periods. Blots were probed with anti-HA and anti-GFP antibodies

4 | CONCLUSION

We identified a shared, maternally inherited, *Ube3A* sequence variant (p.Asn340del) in eight related individuals with a clinical phenotype that does not match the consensus clinical criteria of AS (Williams et al., 1995, 2006). Although all probands showed an apparent intellectual disability ranging from severe to moderate, they showed a milder degree of intellectual disability, were less affected in speech and did not show the ataxia typically seen in individuals with AS. While some notable clinical variation is present in the affected members of this family, the phenotypic differences between the probands carrying the p.Asn340del sequence variant and individuals with AS due to other genetic causes of AS is however substantial, and clinical criteria are not met. In our opinion it is, therefore, not appropriate to classify these patients as "mild, attenuate or atypical Angelman" or "Angelman-like." Patients with a nonclassical AS phenotype have been described in some patients with an imprinting defect (Burger, Kunze, Sperling, & Reis, 1996; Fairbrother et al., 2015; Gillessen-Kaesbach et al., 1999) as well as in AS patients with paternal uniparental disomy (Bottani et al., 1994; Brockmann, Böhm, & Burger, 2002; Fridman, Varela, Nicholls, & Koiffmann, 1998; Gillessen-Kaesbach, Albrecht, Passarge, & Horsthemke, 1995). More recently a milder phenotype was reported for three individuals that have a mutation in the start codon of human UBE3A isoform 1 (p.Met1Thr), which is predicted to result in loss of expression of isoform 1, but not of isoforms 2 and 3. (Sadhwani et al., 2018). Similar to the affected individuals described in this report, the majority of our patients had normal gait and used language spontaneously to express their needs, exceeding developmental skills seen in other individuals with AS. This was also confirmed by the relatively high scores on the Vineland Adaptive Behavior Scales. On a molecular level, individuals with the p.Met1Thr variant lack the most abundantly expressed human isoform 1, which is homologous to the nuclear mouse isoform 3 (Avagliano Trezza, 2019). However, they do express the less abundant isoforms 2 and 3, resulting in low levels of ligase competent UBE3A, which may explain their relatively mild phenotype.

Results from analyses with polymorphic markers provided supporting evidence of p.(Asn340del) being the causative variant in this family. The sequence variant p.Asn340del was identified on the maternally inherited *UBE3A* allele in all affected individuals and was not detected in III.6. Moreover, the unaffected mother (III.1) of two affected brothers, IV.3, and IV.4, was found to carry the p.Asn340del sequence variant on her paternally inherited *Ube3A* allele, confirming the dependency on imprinting.

The p.Asn340del mutation is located in a mutational hotspot in the N-terminal half of UBE3A. Recent evidence suggests that the UBE3A N-terminal domain (where the p.Asn340del variant is located) interacts with the HECT domain and that these intramolecular interactions can modulate the catalytic activity of UBE3A (Mortensen et al., 2015; Sailer et al., 2018). It is conceivable, therefore, that mutations in the N-terminal domain can impact the function of UBE3A. In line with this, previous work of Yi and colleagues has shown that AS-linked missense mutations in this specific region of the UBE3A N-terminal domain can result either in decreased, intrinsic, protein stability, or reduced HHR23A (target) ubiquitination (Yi et al., 2015). Functional analyses of the p.Asn340del (mouse p.Asn337Δ) variant indicated that nuclear localization and ubiquitin protein ligase activity (both auto-ubiquitination and target, Ring1b, ubiquitination) of mUBE3A-iso3$^{N337\Delta}$ were unaffected in our in vitro/in vivo assays (Figure 2c-f). Protein levels of mUBE3A-iso3$^{N337\Delta}$ were comparable to mUBE3A-iso3 when expressed in HEK293T cells (Figure 2g) and we did not observe a difference in intrinsic protein stability in a cycloheximide chase experiment (Figure 2h). While this data indicate that the p.Asn340del (mouse p.Asn337Δ) variant does not have major functional deficits, subtle changes in activity or localization may not be detected in our vitro/in vivo assays. Also, we have tested

the ubiquitination of a single target, Ring1b, whose ubiquitination by the p.Asn340del variant is unaffected, but if ubiquitination of other UBE3A targets is changed remains to be investigated. Despite these limitations, our data indicate that the functional changes caused by the p.Asn340del variant must be subtle, a conclusion that is supported by the relatively mild phenotypes observed in the affected individuals. Further studies are required to clarify how the UBE3A p.Asn340del variant differs from wild-type UBE3A as well as from variants that cause typical AS phenotypes. Such studies may provide more insight into the biochemical function of UBE3A and its role in neurodevelopment.

With the rapidly increasing use of whole-exome sequencing (WES) we expect variants with uncertain meaning due to discrepancy of the phenotype of the patients as opposed to the phenotype known to be associated with mutations in the gene, to expand (Tetreault, Bareke, Nadaf, Alirezaie, & Majewski, 2015). It is, therefore, important to collaborate internationally on the gathering of genotypes and phenotypes. Furthermore, our current work emphasizes that such studies should be accompanied with functional studies of the mutant protein to further understand and delineate the range in clinical phenotype caused by different mutations in UBE3A.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests.

AUTHOR CONTRIBUTIONS

Amber Geerts-Haages, Inge den Besten, and Marlies Valstar visited the patients, conducted interviews and performed clinical examination, studied the medical records and analyzed the clinical data. Ineke van der Burgt examined the family in the 90's and started the linkage analyses. Helger G. Yntema performed linkage analysis and assigned the causal genetic defect in 2002. Hennie Bruggenwirth performed further genetic analysis of the family members. Alice Brooks visited the patients and their families, and conducted genetic counseling. Stijn N. V. Bossuyt generated UBE3A constructs and performed confocal imaging experiments and western blotting. A. Mattijs Punt performed ubiquitination experiments. Marlies Valstar, Ben Distel, and Ype Elgersma supervised the research. Marlies Valstar, Ben Distel, Amber Geerts-Haages, and Stijn N. V. Bossuyt wrote the first draft of the manuscript. All authors reviewed the manuscript.

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