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Severe neurocognitive and growth disorders due to variation in *THOC2*, an essential component of nuclear mRNA export machinery

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Abstract

Highly conserved TREX-mediated mRNA export is emerging as a key pathway in neuronal development and differentiation. TREX subunit variants cause neurodevelopmental disorders (NDDs) by interfering with mRNA export from the cell nucleus to the cytoplasm. Previously we implicated four missense variants in the X-linked *THOC2* gene in intellectual disability (ID). We now report an additional six affected individuals from five unrelated families with two *de novo* and three maternally-inherited pathogenic or likely pathogenic variants in *THOC2* extending the genotypic and phenotypic spectrum. These comprise three rare missense *THOC2* variants that affect evolutionarily conserved amino acid residues and

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reduce protein stability and two with canonical splice-site *THOC2* variants that result in C-terminally truncated THOC2 proteins. We present detailed clinical assessment and functional studies on a *de novo* variant in a female with an epileptic encephalopathy and discuss an additional four families with rare variants in *THOC2* with supportive evidence for pathogenicity. Severe neurocognitive features, including movement and seizure disorders were observed in this cohort. Taken together our data show that even subtle alterations to the canonical molecular pathways such as mRNA export, otherwise essential for cellular life, can be compatible with life, but lead to NDDs in humans.

KEY WORDS

XLID; *THOC2*; mRNA export; protein stability; partial loss-of-function variants

INTRODUCTION

Intellectual disability (ID), characterized by substantial limitations in both intellectual functioning and adaptive behaviour, affects 1-3% of the population starting before the age of 18 years and has significant impact on individuals, families and communities (Vissers, et al., 2016). Individuals with ID are more likely than members of the general population to experience poor physical and mental health, have a lower life expectancy, experience inequalities accessing health care and frequently have limited or no specific therapies for their core symptoms (Bittles, et al., 2002; Hosking, et al., 2016). Both genetic and environmental factors contribute to the development of ID (Milani, et al., 2015). Over 120 of

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the identified >800 ID genes are located on the X-chromosome (Chiurazzi and Pirozzi, 2016; Schwartz, 2015), and diagnosis of X-linked causes of ID remain critically important for accurate genetic counseling of families (Ropers and Hamel, 2005). Dramatic improvements in high-throughput DNA sequencing technologies and analyses software has led to identification of new ID genes and additional variants in the known ID genes (Dickinson, et al., 2016; Vissers, et al., 2016). A systematic review of clinical data suggests that ID affected individuals frequently have comorbid neurological, psychiatric and behavioural disorders (Oeseburg, et al., 2011; Vissers, et al., 2016), and disease variants in different parts of a gene can lead to a broad range of complex neurocognitive disorders (Palmer, et al., 2017; Zhu, et al., 2014). This complexity contributes to heterogeneity in clinical symptoms and indistinct boundaries between syndromic and non-syndromic forms of NDD.

In 2015, we reported genetic, molecular and protein structural data on four missense variants in an X-linked essential gene *THOC2* (MIM# 300957; NM_001081550.1; c.937C>T (p.Leu313Phe), c.1313T>C (p.Leu438Pro), c.2399T>C (p.Ile800Thr) and c.3034T>C (p.Ser1012Pro), RNA not analysed) (Kumar, et al., 2015). The affected individuals had a syndromic NDD, characterized by borderline to severe ID, speech delay, short stature and adult onset truncal obesity (Kumar, et al., 2015). *THOC2* encodes for the THOC2 protein - the largest subunit of the highly conserved TREX (Transcription-Export) mRNA export complex essential for exporting mRNA from the cell nucleus to the cytoplasm (Heath, et al., 2016). The TREX complex is composed of a THO sub-complex (THOC1, THOC2, THOC3, THOC5 and THOC7) and accessory proteins (UAP56, UIF, Aly, CIP29, PDIP3, ZC11A,

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SRRT, Chtop) (Heath, et al., 2016). The TREX complex, besides its canonical role in mRNA export in the mammalian cells, has been shown to play critical roles in gene expression, 3' mRNA processing, stress responses, mitotic progression and genome stability as well as developmental processes such as pluripotency maintenance and hematopoiesis (Yamazaki, et al., 2010). We and others have recently demonstrated that subtle perturbations in mRNA export by gene variants or preferential cytoplasmic aggregation can lead to NDDs (Beaulieu, et al., 2013; Coe, et al., 2014; Kumar, et al., 2015), neurodegeneration (Woerner, et al., 2016) or cancer (Chinnam, et al., 2014; Hautbergue, 2017; Liu, et al., 2015; Viphakone, et al., 2015). These alterations can have tissue-specific effects as TREX subunits are shown to have tissue-specific roles; for example, mouse *Thoc5* and *Thoc1* deficiency interferes with the maintenance of hematopoiesis (Guria, et al., 2011; Mancini, et al., 2010) and testis development (Wang, et al., 2009). Taken together, altered TREX function can have diverse molecular and cellular consequences resulting in a range of diseases. Here we present detailed information on the clinical presentations and functional investigations on an additional eight missense and two splice *THOC2* variants. These data reaffirm and extend our previous findings that *THOC2* variation plays a role in complex neurodevelopmental conditions with the core clinical presentation of ID.

MATERIALS AND METHODS

Molecular and cellular studies

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RNA extraction, RT-qPCR (primers listed in Supp. Table S3), cycloheximide chase, and THOC2 immunofluorescence staining were performed as reported previously (Kumar, et al., 2015). Molecular studies on the *THOC2* exon35:c.4450-2A>G variant were performed using blood DNA and skin fibroblasts derived from the affected individual and his heterozygous carrier mother. Genomic DNA or cDNA (generated by reverse transcribing the fibroblast total RNAs using Superscript III reverse transcriptase; Life Technologies) was amplified with KAPA HiFi PCR Kit (Kapa Biosystems) using hTHOC2-4326F/ hTHOC2-4519-R (Supp. Table S3) at 95°C for 3 min, 35 cycles of 98°C-15sec, 59°C-15sec, 72°C-30sec, incubation at 72°C for 10 min, gel purified (MinElute Gel Extraction kit (Qiagen) and Sanger sequenced using the same primers. For the *THOC2* exon28:c.3503+4A>C, blood gDNA from unaffected father, carrier mother and affected son was amplified with TaKaRa ExTaq using THOC2-F/THOC2-R primers (Supp. Table S3) at 94°C for 2 min, 40 cycles of 94°C-30sec, 60°C-30sec, 72°C-30sec, incubation at 72°C for 5 min. The cDNA was generated by reverse transcribing the white blood cell RNAs using Superscript III reverse transcriptase (Life Technologies) and amplified with TaKaRa ExTaq using THOC2-ex27F/THOC2-ex30R (Supp. Table S3) at 94°C for 2 min, 28 cycles of 94°C-30sec, 60°C-30sec, 72°C-30sec, incubation at 72°C for 5 min. The amplified products were analyzed by Sanger sequencing.

Generation of *THOC2* variant expression constructs

Generation of the wild type Myc-tagged human THOC2 expression plasmid was reported earlier (Kumar, et al., 2015). Briefly, the *THOC2* variants were introduced into the existing pCMV-Myc-THOC2 expression construct by overlap PCR method using the primers listed in

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Supp. Table S3. The variant plasmid sequences were confirmed by Sanger sequencing. Details relating generation of the *THOC2* variant expression constructs are available on request.

Transient expression and Western blotting

For transient expression experiments, HEK293T and HeLa cells were transfected with expression constructs (400 ng pCMV-Myc-THOC2 plasmid and 400 ng pEGFP-C1 plasmid/transfection for stability and cycloheximide assays and 4 µg/transfection for immunofluorescence staining, IF) using Lipofectamine 3000 reagent according to manufacturer's protocol (Life technologies). Twenty-four hours post-transfection, cells were either fixed with 4% formaldehyde for IF or collected and lysed in buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Triton-X 100, 1 mM EDTA, 50 mM NaF, 1× Protease inhibitor/no EDTA and 0.1 mM Na_3VO_4 for western blot assay as reported previously (Kumar, et al., 2015).

***In silico* pathogenicity prediction**

We used CADD v1.3 (includes Phylop, GERP++ & PolyPhen2) (Kircher, et al., 2014), Provean (Choi and Chan, 2015) and ACMG (Richards, et al., 2015) on-line tools for *in silico* prediction of the pathogenicity of different variants (Table 1, Supp. Table S1).

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RESULTS

Identification of *THOC2* variants

We previously implicated four missense *THOC2* variants in 25 individuals with ID and a range of other clinical features (Table 1, Supp. Table S1-2) (Kumar, et al., 2015). We identified an additional five *THOC2* variants (three missense; *de novo* c.2087C>T (p.Thr696Ile), *de novo* c.2138G>A (p.Gly713Asp), maternally-inherited c.3559C>T (p.His1187Tyr), and two splicing-defective; maternally-inherited chrX:122747561 exon35:c.4450-2A>G and chrX:122757634 exon28:c.3503+4A>C; GenBank: NM_001081550.1) variants in a further six affected individuals, including one pair of monozygotic twins (Table 1, Figures 1-2). Whole exome (WES) or whole genome sequencing (WGS) of probands and parents was used to identify the variants that were confirmed by Sanger sequencing of the PCR amplified variant-carrying region of genomic DNA of the parents and affected individuals. The previously unreported *THOC2* variants affect amino acids that are highly conserved (Supp. Figure S1), are absent in gnomAD database and are predicted to be pathogenic based on a number of *in silico* analyses tools (Table 1). We included in our study a *de novo* missense p.Tyr517Cys variant in a female with moderate-severe ID, speech problems, epileptic encephalopathy, cortical visual impairment, and gait disturbances identified using WES as part of the Epi4K Consortium & Epilepsy Phenome/Genome Project (Epi, et al., 2013) (Table 1). We have also collected further, rare and potentially pathogenic variants through international collaboration (Table1, Supp. Table S1-2, Figures 1 and 3, see Supplementary information for methods used for identifying the variants) and performed functional testing on several of these. The following three variants:

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c.229C>T (p.Arg77Cys), c.3034T>C (p.Ser1012Pro) and c.3781A>C (p.Asn1261His) showed no clear evidence of altered stability of variant THOC2 proteins in our cell-based assay. The reported variants have been submitted to ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/accession numbers SCV00680065-SCV000680074).

Clinical presentations

The clinical features of the five previously unreported affected individuals with (likely) pathogenic *THOC2* variants, aged between 3 and 12 years, and the 10 year old female with *de novo* p.Tyr517Cys variant are summarised in Table 2 and photographs, when available, are shown in Figure 2. Detailed clinical information is available in the supplementary data. Each individual clinical centre used local diagnostic criteria for determining degree of ID and diagnoses of co-morbidities. ID was universal and at least moderate in severity: 2/7 were non-ambulatory and 3/7 non-verbal. Behavioural problems were reported in four individuals, with one meeting diagnostic criteria for autism spectrum disorder. Additional neurological features were common. Four of the seven had infantile hypotonia and two of the seven had tremor. The monozygotic twins (individuals 3-4) had a tendency to toe walking, which was considered behavioural as it was not associated with neurological signs of lower limb spasticity. Confirmed seizure disorder was only present in the affected female (individual 7) but suspected in individual 2. Neuro-radiological studies were performed in five individuals: this was within normal limits for three individuals, with neuroanatomical differences reported in two. Individual 2 had complex neuroanatomical findings (see supplementary clinical description and Supp. Figure S2A) including changes in cortical gyral morphology, which in

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the inferior temporal lobes appeared finely nodular, as well as hypoplasia of the corpus callosum and reduced brainstem volume and individual 5 had mild dilatation of the lateral ventricles, mildly delayed myelination and an abnormal white matter lesion in the periventricular area close to the anterior horn (Supp. Figure S2B). Growth abnormalities were common including low birth weight (3/7), microcephaly (2/7) and short stature (2/7). Facial features are shown in Figure 2. Appropriate consent for reporting variants, clinical data and photographs of the affected individuals was obtained from their parents or legal guardians. The research has been approved by the Women's and Children's Health Network Human Research Ethics Committee in Adelaide, Australia.

THOC2 variant protein localisation and stability

Without access to affected individuals' derived cells, we generated Myc-tagged THOC2 missense variant expression constructs to determine protein stability and localisation. The THOC2 protein stability was determined in HEK293T cells and localisation in both the HEK293T and HeLa cells. Total protein lysates of HEK293T cells ectopically-expressing the wild type or variant Myc-THOC2 proteins were western blotted for THOC2, EGFP and β-Tubulin. We used HEK293T cells expressing Myc-p.Ile800Thr THOC2 as a control for protein stability assay as this variant is shown to cause reduced protein stability (Kumar, et al., 2015). The results showed reduced stability of p.Tyr517Cys, p.Thr696Ile, p.Gly713Asp and p.His1187Tyr THOC2 compared to the wild type protein (Figure 3A). Presence of comparable levels of EGFP in the cells transfected with different expression constructs indicated that the reduced levels of THOC2 protein were not due to difference in transfection

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efficiency (Figure 3A). We also determined the turnover rate of Myc-p.Tyr517Cys THOC2 protein by cycloheximide chase. For this assay, the HEK293T cells transfected with pCMV-Myc-WT or pCMV-Myc-p.Tyr517Cys THOC2 and pEGFP-C1 transfection control plasmids were cultured in presence of translation inhibitor cycloheximide for different durations and western blotted for THOC2, EGFP and β-Tubulin. The results showed that p.Tyr517Cys THOC2 turnover rate was 3h compared with 8h for the wild type protein (Figure 3B). THOC2 variant proteins, similar to the wild type, were mainly localised to the nucleus in both the HEK293T and HeLa cells (Supp. Figure S3).

THOC2* splice variant: exon35:c.4450-2A>G, p.Arg1483fs52

Sanger sequencing of amplified target region from affected son and mother's blood genomic DNA showed that the affected boy inherited chrX:122747561 exon35:c.4450-2A>G variant from his unaffected heterozygous carrier mother (Figure 4C). A -2 A>G change in the intron-exon splicing site boundary (acceptor AG) is predicted to abolish splicing (Ohno, et al., 2018). To validate this possibility, we generated skin fibroblast cultures from the heterozygous carrier mother and the affected son. We PCR amplified their fibroblast cDNAs using primers with binding sequences located within exon 34 and 35. Amplification of a 194 bp DNA fragment from the mother indicated normal splicing but a 537 bp product from the affected son indicated retention of the intron located between these exons (Figure 4A-B). We confirmed this result by Sanger sequencing of the PCR products generated from genomic DNA that showed presence of A/G nucleotides in the carrier mother but only G (A>G) nucleotide in the affected son (Figure 4C). The cDNA sequence showed presence of

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normally-spliced mRNA in the mother but retention of intronic sequence upstream of the exon 35 in the affected son indicating defective splicing due to presence of -2 G variant at the intron-exon 35 junction sequence (Figure 4C). The presence of normally-spliced mRNA in the unaffected mother is consistent with X-inactivation (94% skewing) of the variant allele in her fibroblasts. We predicted that a retention of intron between exon 34-35 in the affected fibroblasts would result in loss of 110 C-terminal amino acids of the 1593 wild type THOC2 protein (that is, 1483 amino acids); however, overall the variant protein would be 58 amino acid smaller as it would now be a 1535 amino acid protein comprised of 1483 amino acids of wild type THOC2 and 52 translated from intronic sequence in the defective mRNA (Figure 4A). Consistent with our prediction, the western blot data showed presence of a slightly smaller THOC2 protein band in the affected son's fibroblasts than his unaffected mother. Many independent western blot runs showed presence of two closely-located THOC2 bands - similar to the fruit fly THO2 (Rehwinkel, et al., 2004) - in the unaffected mother but a single highly intense band in the affected son's fibroblasts (Figure 4D). The observed difference in levels of THOC2 protein was post-translational as we found comparable amounts of *THOC2* mRNA, as assayed by real time RT-qPCR, in the mother and son (Figure. 4E). Finally, we observed no difference in THOC2 localisation in fibroblasts of the affected son and his unaffected mother (Figure 4F).

***THOC2* splice variant: exon28:c.3503+4A>C, p.Gly1168fs7* and normal 1593 aa protein**

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For the second splice variant chrX:122757634 exon28:c.3503+4A>C, molecular studies were performed on the white blood cells of the unaffected father, carrier mother and the male proband. Sanger sequencing of target region amplified from the unaffected father and mother, and affected son's genomic DNA showed that the affected son inherited the A>C change from his unaffected carrier mother who had A/C nucleotides at this position (Figure 5). The intronic nucleotide change A>C at +4 position of the 5' exon-intron donor splicing site sequence is predicted to cause aberrant splicing (https://www.med.nagoya-u.ac.jp/neurogenetics/SD_Score/sd_score.html). To confirm this possibility, we amplified cDNA generated by reverse transcribing blood RNA of the father, mother and the affected son using primers located within exon 27 and exon 30 (Figure 5A; Supp. Table S3). Interestingly, whereas a 491 bp PCR product was observed in highly skewed carrier mother (98:2%) and normal father, 491 bp and 634 bp PCR products were detected in the affected son. A 491 bp amplified product indicated normal splicing in the mother and father, and 491 bp and 634 bp bands suggested partially-defective splicing in the affected son. Amplification of a 634 bp instead of a 994 bp fragment that would have resulted from a complete retention of intron between exon 28-29 indicated aberrant splicing event in the affected son (Figure 5). Sanger sequencing of 491 bp and 634 bp PCR products from the mother, father and son confirmed normal splicing in the mother and father and aberrant splicing in the affected son. The sequence showed retention of a 143 bp instead of complete 503 bp fragment due to activation of a cryptic splice site within the intron between exon 28-29 in the son (Figure 5). Retention of 143 bps from intron between exon 28-29 in the mRNA is predicted to result in a 1175 amino acid truncated THOC2 protein containing 1168 wild type amino acids and 7 amino acids from the translation of the intronic sequence retained in the defective mRNA.

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This aberrant product would be present in addition to the wild type 1593 amino acid protein from the normally-spliced mRNA in the affected son.

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Discussion

Here we present detailed clinical information, and molecular and functional studies, on five previously unreported *THOC2* variants in six affected males (two *de novo* variants and one maternally inherited variant in monozygotic twins) and on one affected female with a previously reported *de novo* p.Tyr517Cys variant. We present evidence that extends the genotypic spectrum beyond the four *THOC2* missense variants that we reported previously (Kumar, et al., 2015) by including two intronic variants that affect splicing, and four missense variants that affect protein stability in a cell-based assay system. According to ACMG criteria they were classified as pathogenic or likely pathogenic (Table 1) (Richards, et al., 2015). These findings, along with the four missense variants reported earlier (Kumar, et al., 2015), add to the existing evidence that alterations in essential mRNA export pathway cause NDDs (Amos, et al., 2017; Beaulieu, et al., 2013; Kumar, et al., 2015).

We confirm that the core clinical feature of *THOC2*-related disorder in hemizygous males is ID, with several individuals having additional features including behavioural disorders, hypotonia, gait disturbance, tremor, low birth weight, short stature, microcephaly and variable neuroimaging findings. Although the range of neurodevelopmental features is similar, our original cohort contained males with ID in the mild or borderline range of

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intellectual functioning (Kumar, et al., 2015), whereas, all individuals in this cohort have ID which is at least in the moderate range. Individuals 2 and 6 had neurological signs which could be consistent with cerebellar dysfunction including tremor and a broad-based gait for individual 2 and nystagmus, tremor and an ataxic broad-based gait for individual 6, in the absence of significant cerebellar abnormalities on MRI. This is interesting given the female patient with knockdown of THOC2 function due to a *de novo* X;8 translocation that created a *PTK2-THOC2* fusion had congenital cerebellar hypoplasia and prominent cerebellar signs with mild intellectual disability (Di Gregorio, et al., 2013). We used computerized face-matching technology to specifically evaluate the cohort to assess if a characteristic facial gestalt was evident across individuals with pathogenic or likely pathogenic variants across our original and this expanded clinical cohort (Supp. Figure S4) (Dudding-Byth, et al., 2017). Although a clearly recognizable facial gestalt was not obvious, there are some similarities. The facial gestalt spectrum associated with *THOC2* pathogenic variants will continue to emerge as more individuals are reported.

As was the case in our original cohort, heterozygous mothers were clinically unaffected, and, where available, X-chromosome inactivation (XCI) was highly skewed (Table 1). In contrast individual 7, with a *de novo* missense variant (p.Tyr517Cys) is a female with a particularly severe neurocognitive presentation. This is consistent with other reported severely affected females with *de novo* variants in X linked genes (de Lange, et al., 2016; Palmer, et al., 2016; Snijders Blok, et al., 2015; Zweier, et al., 2014). Unfortunately, we did not have access to individual 7's genomic DNA to test XCI status.

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A range of protein-protein interactions are required for mRNA export (Chi, et al., 2013). Proteins with altered stability (Hirayama, et al., 2008), localization (Beaulieu, et al., 2013) (e.g., THOC6 p.Gly46Arg implicated in syndromic ID) or interaction (Chi, et al., 2013) can impact mRNA export and consequently disrupt normal cell function. We did not observe mislocalization of the THOC2 variant proteins in cultured cells and did not test alterations in their interaction with the other known or unknown TREX proteins. However, reduced levels of a number of new (p.Tyr517Cys, p.His1187Tyr, p.Thr696Ile, p.Gly713Asp) and published (p.Leu438Pro, p.Ile800Thr, (Kumar, et al., 2015) missense THOC2 variant proteins are due to impaired protein stability or reduced levels of normal mRNA due to aberrant splicing (exon28:c.3503+4A>C). We also noted increased stability of p.Asn1261His THOC2 protein. We and others have shown that THOC2 controls TREX function by maintaining the stability of THOC1, 3, 5 and 7 subunits (Chi, et al., 2013; Kumar, et al., 2015). Reduced levels of THOC2 missense variant proteins are most likely due to enhanced proteasome-mediated degradation as THOC2 is ubiquitinated (Lopitz-Otsoa, et al., 2012). THOC2 depletion has been reported to have different consequences in diverse organisms. For example, shRNA-mediated Thoc2 knockdown leads to significant increase in length of neurites in cultured rat primary hippocampal neurons (Di Gregorio, et al., 2013) although effects on neurons with persistently reduced THOC2 variant proteins in the affected individuals may be different and *C. elegans thoc2* knockouts, that are completely immobile, slow-growing, sterile, have functional defects in specific sensory neurons and die prematurely (Di Gregorio, et al., 2013). *D. rerio Thoc2* is essential for embryonic development (Amsterdam, et al., 2004) and in *D.*

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melanogaster S2 cells *Thoc2* knockdown inhibits mRNA export and cell proliferation (Rehwinkel, et al., 2004). THOC2 depletion also results in chromosome alignment, mitotic progression and genomic stability in human HeLa cells (Yamazaki, et al., 2010). Finally, *Thoc2* and *Thoc5* knockdown experiments have shown their role in regulation of embryonic stem cell (ESC) self-renewal (Wang, et al., 2013).

Both the affected individuals carrying the splice-variants presented with severe neurocognitive features. The exon35:c.4450-2A>G and exon28:c.3503+4A>C *THOC2* splice variants present interesting biological scenarios; the former resulting in a 1535 amino acid truncated protein that is present at higher level and the latter with both normal (albeit potentially much reduced) and a 1175 amino acid truncated THOC2 protein. We postulate that the clinical outcomes in the exon35:c.4450-2A>G individual are caused by partial loss of function due to loss of 110 amino acid C-terminal region and accumulation of the truncated THOC2 protein. However, pathogenicity in exon28:c.3503+4A>C affected individual is most likely caused by reduced levels of normal and potential dominant-negative effects of the C-terminally truncated THOC2 protein. That reduced THOC2 protein levels are associated with ID and other clinical symptoms is emerging as a frequent theme; e.g., due to reduced THOC2 protein stability caused by missense variants (see above) or aberrant splicing . Indeed reduced THOC2 levels are shown to destabilize the TREX complex in humans (Chi, et al., 2013; Kumar, et al., 2015) and removal of any THO subunit causes destabilization of other TREX components in yeast (Pena, et al., 2012).

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Systematic functional analysis of the Tho2 C-terminal RNA binding region in yeast provides interesting explanation as to how the truncated THOC2 protein can perturb normal mRNA export function in human cells (Pena, et al., 2012) (Figure 6). The data showed that whereas ΔTho2 yeast strain does not grow at 37°C (restrictive temperature), Tho2$\Delta_{1408\text{-}1597}$ and Tho$\Delta_{1271\text{-}1597}$ growth is considerably reduced, suggesting that C-terminal 1271-1597 amino acids are required for cell survival at restrictive temperature (Pena, et al., 2012). If the exon28:c.3503+A>C variant caused complete splicing defect retaining intron between exon 28-29 in all mRNAs, the cells would translate only 1175 amino acids (with 1168 normal) THOC2 protein; essentially lacking the C-terminal region encompassing the RNA binding domain (RBD) that when deleted in yeast Tho$\Delta_{1271\text{-}1597}$ strain restricts its growth at 37°C. However, the affected boy carrying a single allele of the exon28:c.3503+A>C *THOC2* variant, although with severe clinical symptoms, is alive. This could be explained by presence of reduced levels of THOC2 protein produced from translation of about 2/3rd normally-spliced mRNA in the affected white blood cells. Taken together, clinical outcomes in the affected boy may be due to perturbed mRNA export caused by reduced levels of THOC2 protein and perhaps also C-terminally truncated THOC2 protein translated from about 1/3rd aberrantly spliced mRNAs that retain a part of intron sequence between exon 28-29.

We also identified a set of previously unreported *THOC2* missense variants that, according to ACMG criteria, are variants of uncertain clinical significance (VOUS) (Supp. Table S1): namely p.Arg77Cys; p.Ser1108Leu; p.Arg1121Gly and p.Asn1261His. Nevertheless, these variants have supportive evidence pointing towards potential pathogenicity as they are rare

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(absent from ExAC/gnomAD databases of reference individuals) (Lek, et al., 2016), affect highly evolutionarily conserved amino acid residues, are predicted to be pathogenic by *in silico* analyses and are within the clinical presentations spectrum of those seen in individuals with confirmed *THOC2*-related ID. However, they lack supportive evidence from our existing functional assays. These variants may still have a detrimental effect on THOC2 function due to altered protein structure impacting protein-RNA and/or protein-protein interactions with known or unknown TREX subunits [E.g., (Boehringer, et al., 2017)]. The challenge of proving causality for previously unreported missense variants in NDD genes is well recognized and speaks to the need for ongoing intertwined clinical and research efforts to clarify causality of VOUS (Wright, et al., 2018). We therefore report detailed variant and clinical data (see Supp. materials, Supp. Table S1-S2 and Supp. Figure S4) with the intention of alerting researchers and clinicians to these variants, as future studies, for example identification of their recurrence in affected individuals with overlapping clinical phenotypes or pathophysiological investigations, may help clarify their clinical significance.

THOC2 is ubiquitously expressed in all human tissues (Thul, et al., 2017) and more specifically in the developing and mature human brain (Johnson, et al., 2009; Kumar, et al., 2015; Uhlen, et al., 2015) and mouse brain, with higher abundance in frontal cortex and cerebellum (Di Gregorio, et al., 2013; Kumar, et al., 2015). THOC2 is an essential mRNA export factor as its siRNA-mediated depletion results in almost complete retention of mRNAs in the cell nucleus (Chi, et al., 2013), potentially toxic to the cell. These data are consistent with the findings that *THOC2* is a highly-constrained gene (Samocha, et al., 2014) and *THOC*

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(e.g. *THOC1*, *3*, *5*, *6* and *7*) genes are essential for cell survival (Blomen, et al., 2015). Taken together, as *THOC2* knockout cells will not survive due to complete mRNA nuclear retention, we predict that the identified *THOC2* variants represent partial loss-of-function that disrupt normal mRNA export in neuronal and possibly other cell types, potentially causing variable clinical presentations.

TREX complex couples transcription and mRNA biogenesis with nuclear mRNA export, and has emerged as an essential pathway in embryogenesis, organogenesis and differentiation (Heath, et al., 2016). For example, Thoc2 and Thoc5 selectively bind and regulate export of mRNAs (e.g. *Nanog*, *Sox2*, *Esrrb*, and *Klf4* mRNAs) involved in maintenance of pluripotency of mouse ESCs (Wang, et al., 2013) and Thoc5 in maintenance of hematopoiesis and HSP70 mRNA export (Katahira, et al., 2009; Mancini, et al., 2010). Mouse modeling shows that both *Thoc1* and *Thoc5* knockouts are embryonic lethal (Mancini, et al., 2010; Wang, et al., 2006). However, *Thoc1* and *Thoc5* expression in a range of developing and adult tissues may indicate that the two genes have a more essential role in early embryonic development compared to less stringent requirement during later stages of embryonic or adult development (Mancini, et al., 2010; Wang, et al., 2006); a functional pattern most likely followed by the *THOC2* gene. Essentiality of *THOC2* gene indicates that *THOC2* knockout will also be lethal. However, reduced levels or perturbed functionality can lead to a range of NDD phenotypes as observed for a cohort of THOC2 variants identified by us. It is now well-established that development of brain depends on tightly regulated and complex sequence of events involving neuronal and glial cell proliferation, migration and

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maturation (Chiurazzi and Pirozzi, 2016). Therefore, it is not surprising that our *THOC2* variant data and published work (Dickinson, et al., 2016) provides strong evidence that even subtle alterations to the canonical molecular pathways such as mRNA export, otherwise essential for cellular life, can be tolerated but at a cost of a NDD.

In summary, we present detailed clinical data on seven individuals with *THOC2*-associated ID caused by both missense and splice variants that meet ACMG criteria for (likely) pathogenicity. They have a core phenotype of ID, and common findings of behavioural disorders, infantile hypotonia, gait disturbance and growth impairment, similar to the affected males with THOC2-associated ID we previously reported (Kumar, et al., 2015). Other than the affected female with a *de novo* missense variant, heterozygote carrier females are typically unaffected. We also present data on five individuals with four previously unreported rare missense variants that show clinical overlap with our core group, but where convincing evidence for causality is still required. The significance of these variants may be clarified as additional individuals with *THOC2* variants are reported. We have also 'adopted' *THOC2* on the Human Disease Gene (HDG) Website Series (http://humandiseasegenes.nl/thoc2) in an effort to continue to explore the phenotypic-genotypic spectrum for THOC2-related ID.

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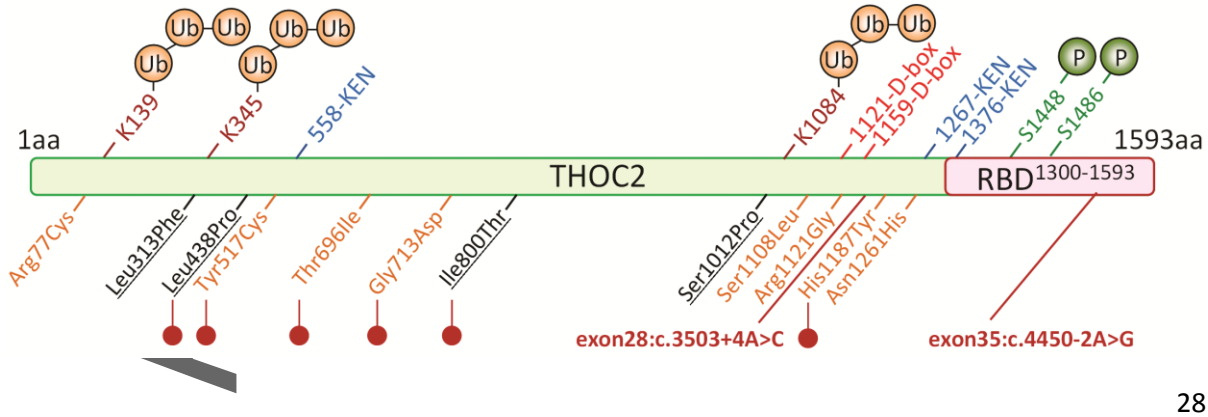
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Figure legends

FIGURE 1 Location of variant amino acids and structural features in THOC2 protein. Ubiquitinated (K139 and K1084 (Kim, et al., 2011) and K345 (Lopitz-Otsoa, et al., 2012; Wagner, et al., 2011)), phosphorylated (S1448 and 1486 (Olsen, et al., 2006)) amino acid residues, potential RNA binding domain (RBD) and destruction box (D-box) and KEN box sequences that interact with the Anaphase Promoting Complex/Cyclosome (APC/C) for protein ubiquitination and subsequent destruction by the proteasome (Morgan, 2013) are shown. Unreported (orange) and published (black: (Kumar, et al., 2015)) missense variants effecting THOC2 protein stability are marked with red lollipops. The positions of two splice variants are shown in red.

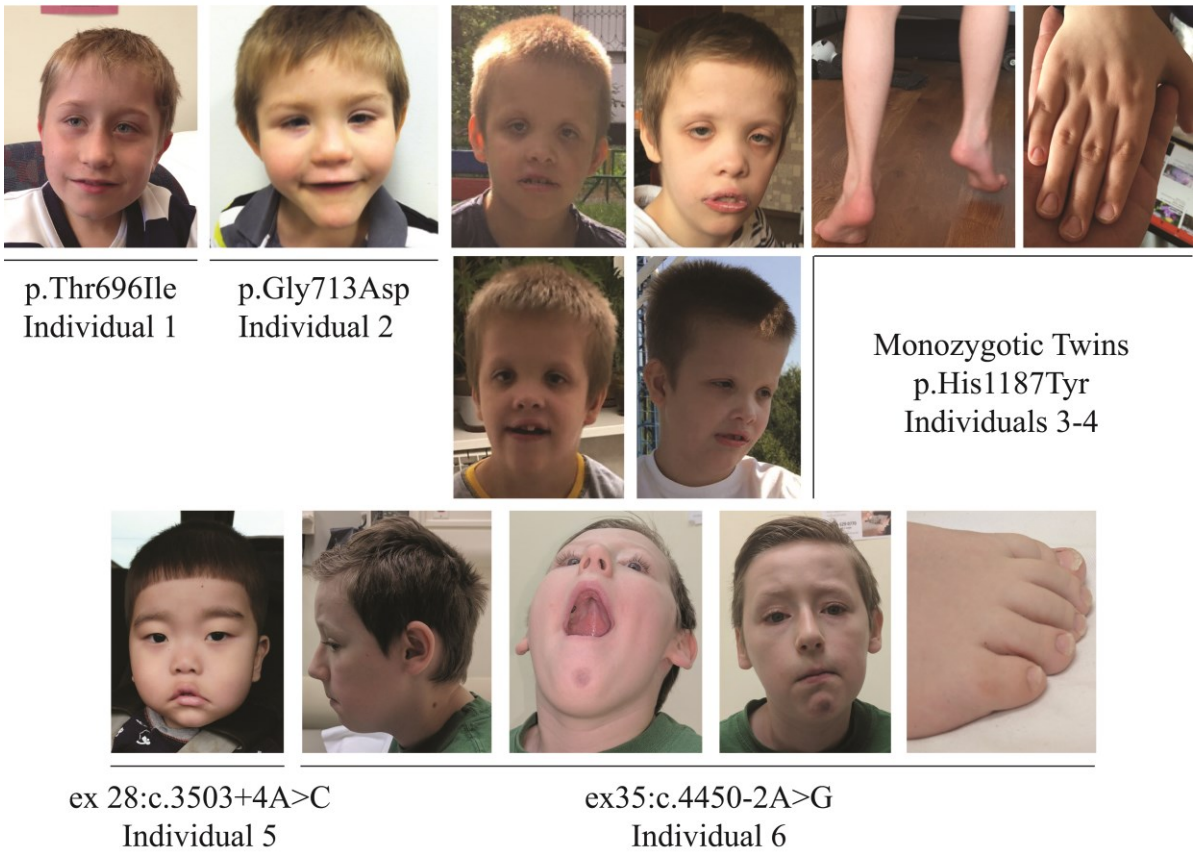
Figure 1



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FIGURE 2 Front and side facial views of the affected individuals with *THOC2* variants.

Figure 2



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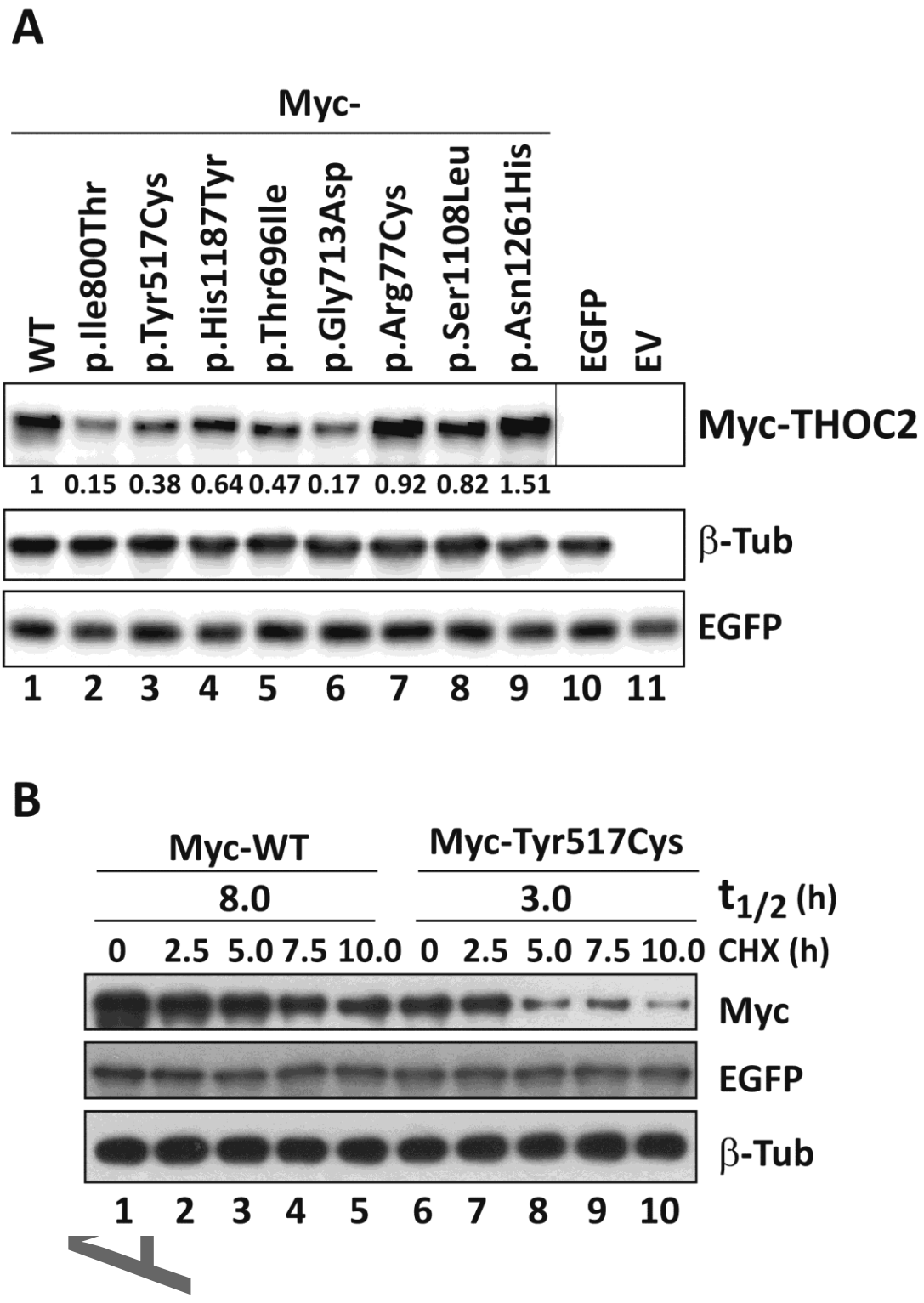
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FIGURE 3 Functional testing of THOC2 missense variants. **A:** THOC2 variant protein stability is reduced in HEK293T cells. pCMV-Myc-THOC2 wild-type or variant expression constructs and pEGFP-C1 plasmid (transfection control) were transfected into HEK293T cells. Total protein lysates of cells 24hr post-transfection were analysed by western blotting with mouse anti-Myc (clone 9E10; Sigma), mouse anti-EGFP (clones 7.1 and 13.1; Roche) and rabbit anti-β-tubulin (loading control; Abcam) antibodies. pCMV-Myc-THOC2 p.Ile800Thr construct expressing the p.Ile800Thr protein shown to have reduced stability was used as a control (Kumar, et al., 2015). Western blot signals were quantified using ImageJ software. Averages of the Myc-THOC2 proteins normalised to the housekeeping β-tubulin signal from two independent runs are shown. **B:** Myc-p.Tyr517Cys THOC2 protein half-life is substantially reduced in HEK293T cells. pCMV-Myc-THOC2 or pCMV-Myc-THOC2-p.Tyr517Cys expression constructs and pEGFP-C1 plasmid (transfection control) were transfected into HEK293T cells. Next day the cells were cultured in the presence of 100 μg/ml translation inhibitor cycloheximide and harvested at the time points shown. Total protein lysates were analysed by western blotting with mouse anti-Myc, mouse anti-EGFP and rabbit anti-β-tubulin (loading control) antibodies.

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Figure 3



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FIGURE 4 Exon35:c.4450-2A>G variant abolishes splicing of intron between exons 34-35. **A:** Part of the *THOC2* gene showing location of the A/G nucleotide in the heterozygous carrier mother and A>G splice variant in the affected son. The C-terminal part of the 1593 amino acid wild type and 1535 amino acid (that contains 1483 normal and 52 amino acids coded by the unspliced intron) THOC2 protein in the affected boy are also shown. **B:** Gel showing a 194 bp RT-PCR product from the normally-spliced mRNA of the heterozygous carrier mother and a 537bp product from defective splicing of mRNA causing retention of an intron between exon 34-35 in the affected son. RT-PCR products from total RNA isolated from passage 3 (lanes 1-2) and 5 (lanes 3-4) fibroblasts. Location of the forward and reverse primers within exons 34 and 35 is shown. **C:** Sanger sequencing chromatograms of PCR products amplified from genomic and cDNA of the affected son and his heterozygous carrier mother using primers located within exon 34 and 35. Genomic DNA around the Exon34-Intron-Exon35 region is shown. **D:** Western blot showing THOC2 protein in the affected son and his carrier mother's skin fibroblasts. TREX subunit UAP56 was used as a loading control. **E:** RT-qPCR showing levels of the THOC2 mRNA in the affected son and his carrier mother's skin fibroblasts. **F:** Immunofluorescence detection of THOC2 in skin fibroblasts of the unaffected mother and affected son.

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Figure 4

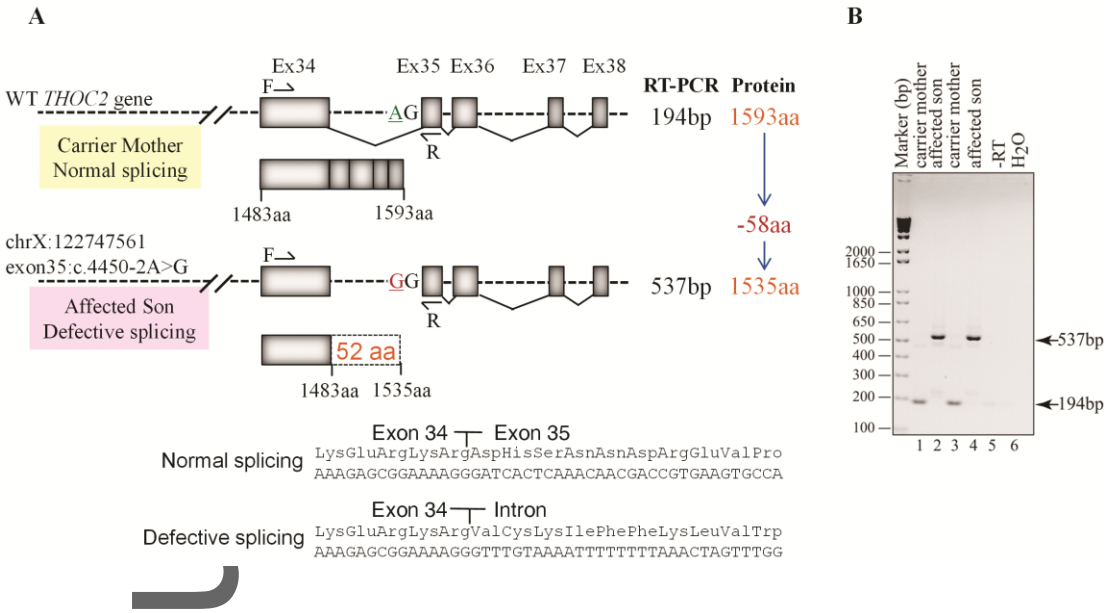
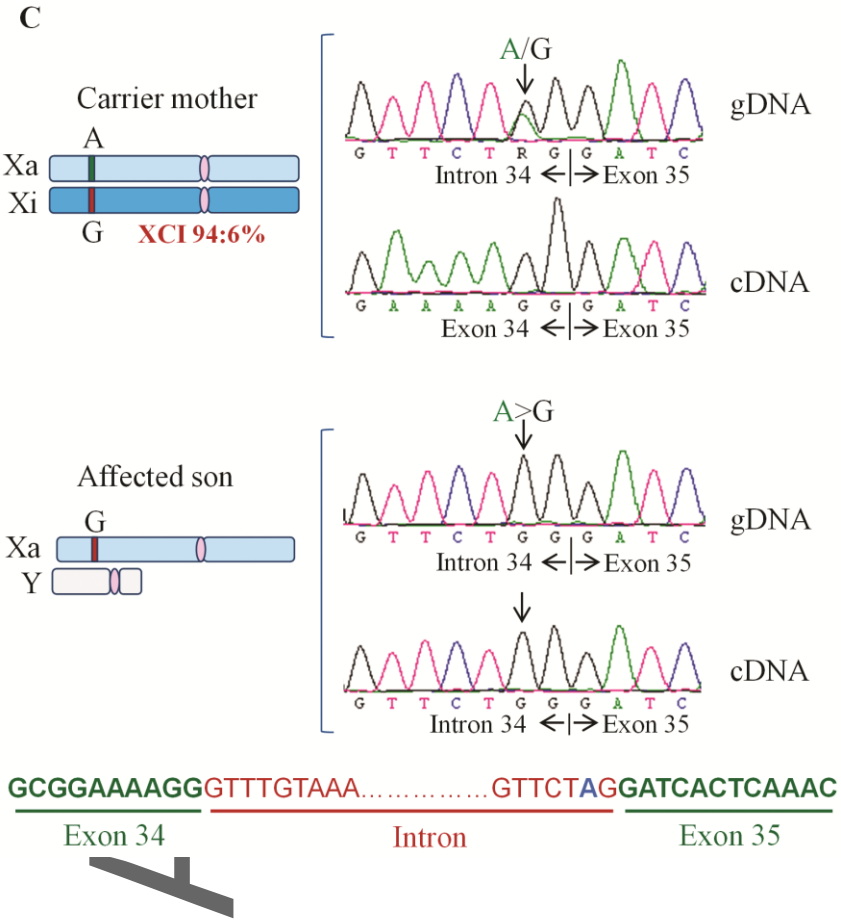


Figure 4



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Figure 4

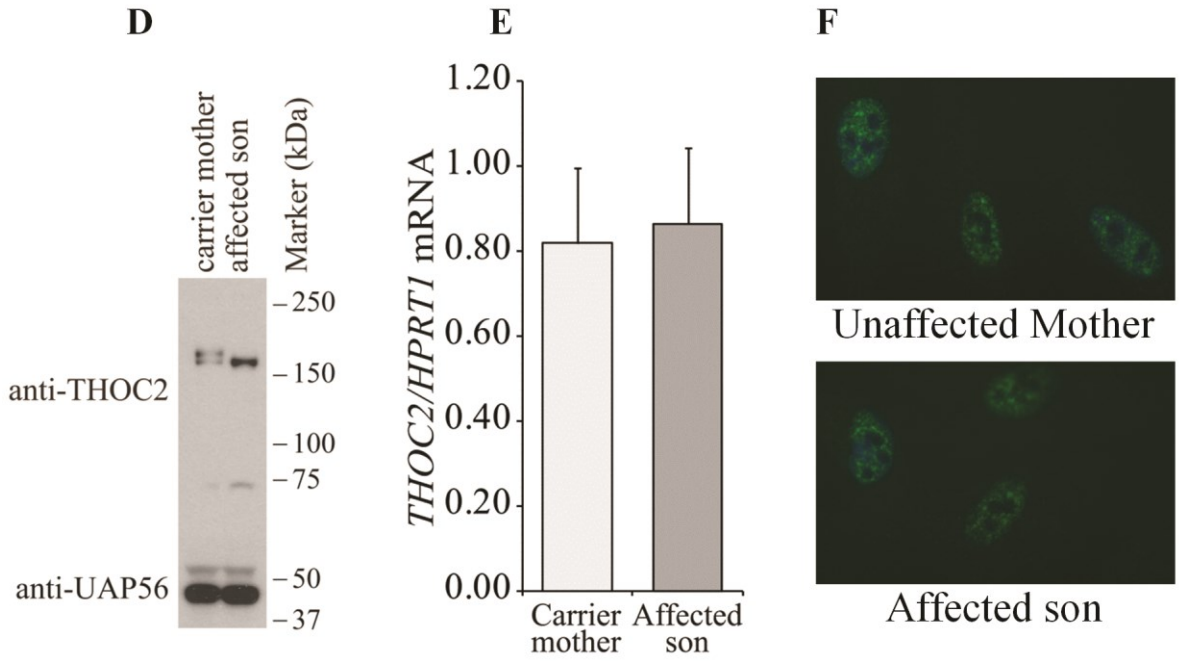


FIGURE 5 Exon28:c.3503+4A>C variant causes aberrant splicing of intron between exons 28-29 **A:** Part of the *THOC2* gene showing location of the A/C nucleotide in the heterozygous carrier mother and A>C splice variant in the affected son. The C-terminal part of the 1593 amino acid wild type and 1175 amino acid (that contains 1168 normal and 7 amino acids coded by the unspliced intron) THOC2 protein in the affected son are also shown. **B:** Gel showing a 491 bp RT- PCR product from the normally-spliced heterozygous carrier mother and unaffected father, and 491 bp and 634 bp (retaining 143 bp of the 503 bp intron between exons 28-29) RT-PCR products derived from the normally and aberrantly spliced mRNAs, respectively, in the affected son. Location of the forward and reverse primers within exons 27 and 30 is shown. **C:** Sanger sequencing chromatograms of PCR

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products amplified using primers located within exons 27 and 30 from genomic and cDNA of unaffected father and mother and the affected son.

Figure 5

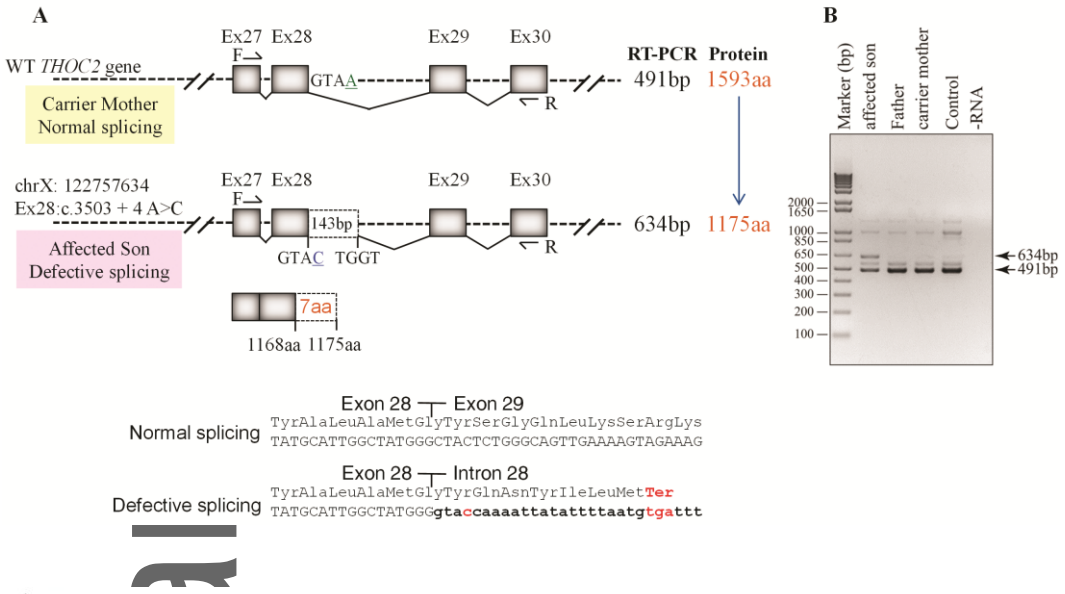
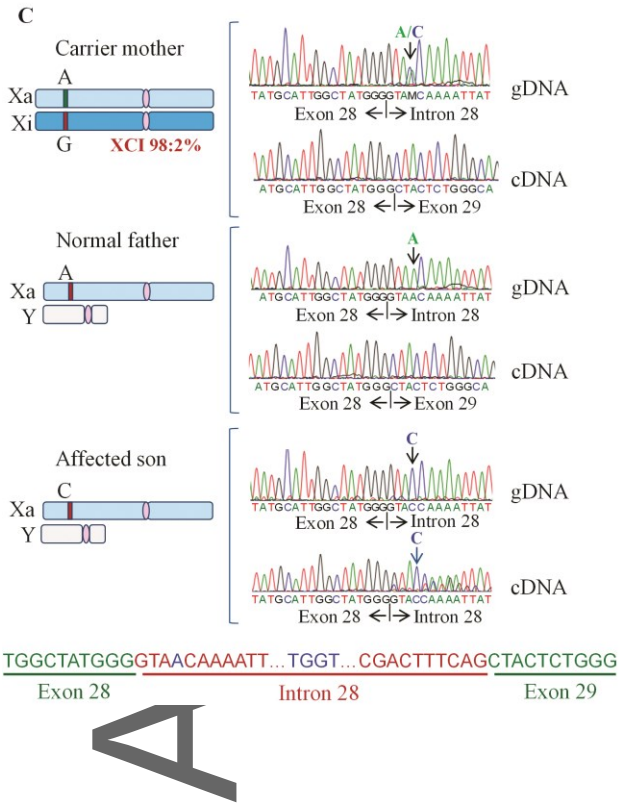


Figure 5

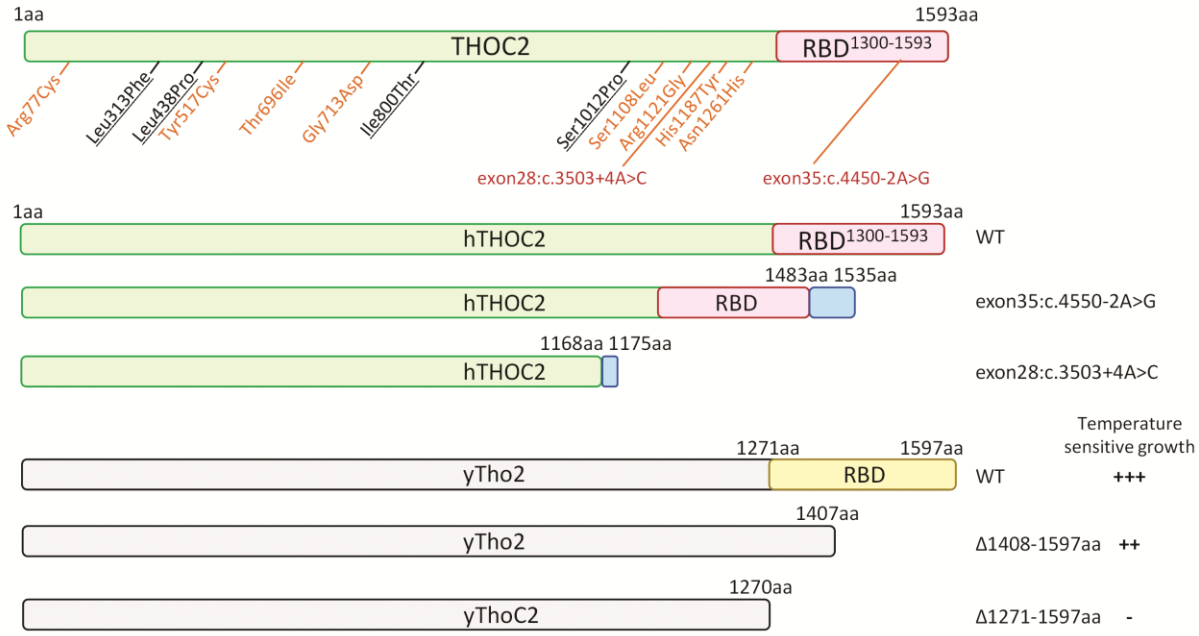


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FIGURE 6 Summary of truncated human THOC2 proteins translated from aberrantly-spliced mRNAs and functional outcomes of yeast C-terminal Tho2 deletion strains (Pena, et al., 2012). Blue boxes depict the 52 and 7 amino acids coded by unspliced intron sequences of exon35:c.4550-2T>C and exon28:c.3503+4A>C variants, respectively. WT, +++ = normal, Δ1408-1597aa, ++ and Δ1271-1597aa = reduced growth at restrictive temperature.

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Figure 6



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TABLE 1 Detailed description of the *THOC2* variants with supporting molecular evidence.

	Individual	From	Method of identification	Position hg19	NM_001081550	Mode of inheritance	CADD	Provean score	Provean prediction	GERP++	Phylop	gnomAD frequency	Polyphen2*	Variant plasmid tested	Reduced Protein Stability	ACMG pathogenicity classification**
Missense		Kumar et al 2015 AJHG	X-Chr Exome sequencing	122799566	c.1313T>C: p.Leu438Pro	Maternal inheritance	28.1	-6.08	Deleterious	5.7	1.902	absent	D	YES	YES	LP
	1	Australia	Trio WES	122767853	c.2087C>T: p.Thr696Ile	De novo	27.4	-5.47	Deleterious	5.03	0.963	absent	D	YES	YES	DP
	2	USA	WES	122766890	c.2138G>A: p.Gly713Asp	De novo	31	-4.69	Deleterious	5.73	2.412	absent	D	YES	YES	DP
		Kumar et al 2015 AJHG	X-Chr Exome sequencing	122765621	c.2399T>C: p.Ile800Thr	Maternal inheritance	23.9	-4.01	Deleterious	5.97	2.016	absent	P	YES	YES	LP
	3-4	Canada/Germany/Russia Identical twins	WES	122757079	c.3559C>T: p.His1187Tyr	Maternal inheritance; Mother skewed (99.9:0.1%)	23.1	-5.07	Deleterious	6.07	2.571	absent	P	YES	YES	LP
Splice	5	Japan	Trio WES	122757634	Exon28: c.3503+4A>C	Maternal inheritance; Mother skewed 98:2%	10.8	N/A	N/A	5.57	1.86	absent	N/A	N/A	ND	LP

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	6	Canada	WES	122747561	Exon35: c.4450-2A>G	Maternal inheritance; Mother skewed 94:6%	23.7	N/A	N/A	5.25	1.735	absent	N/A	Fibroblasts of the affected male proband and carrier mother	NO	LP
Missense	7	USA Epi4K Consortium & Epilepsy Phenome/ Genome Project; Nature 501:217-221, 2013	WES	122778639	c.1550A>G: p.Tyr517Cys	De novo	26.6	-7.87	Deleterious	5.84	1.955	absent	D	YES	YES	DP

*Probably damaging, D; Possibly damaging, P; N/A, not applicable; ND, not determined; ***De novo* Pathogenic, DP; Likely Pathogenic, LP

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TABLE 2 Summary of clinical data of *THOC2* variants with supporting molecular evidence.

Individual	**(likely) pathogenic**						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Variant details	c.2087C>T: p.Thr696Ile	c.2138G>A: p.Gly713Asp	c.3559C>T: p.His1187Tyr Twin 1	c.3559C>T: p.His1187Tyr Twin 2	Exon28: c.3503+4A>C	Exon35:c.4450-2A>G	c.1550A>G: p.Tyr517Cys
Gender	Male	Male	Male	Male	Male	Male	Female
Age (years)	12	5	7	7	3	10	10
Perinatal features							
Gestation (weeks)	36	37	37	37	37	41	NA
Low birth weight (<2.5kg)	Yes	No	Yes	Yes	No	No	NA
Birth weight (g)	2000	2650	1990	2420	3018	4365	NA
Neurologic features							
Intellectual disability	Severe	Mod+	Mod+	Mod+	Severe	Severe	Profound
Speech delay	Yes, single words, signs	Yes, non-verbal	Yes	Yes	Yes, non-verbal	Yes, nasal dysarthria	Yes, non-verbal
Hypotonia	No	Yes	NA	NA	Yes	Yes, central hypotonia	Yes
Spasticity	No	No	No	No	No	Yes- appendicular spasticity	No
Hyperkinesia	No	No	Yes	Yes	Yes	No	No
Tremor	No	Yes, intermittent	No	No	No	Yes	No
Epilepsy	No	Suspected	No	No	No	No	Yes, epileptic encephalopathy

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Gait disturbances	No	Yes, gait/balance problems	Yes, toe walking*	Yes, toe walking*		Non ambulatory	Yes, ataxia/broad based gait	Non ambulatory
Behaviour problems	No	Yes	Yes	Yes		Yes, ASD	No	NR
Anxiety	No	No	No	No		No	No	NR
Depression	No	No	No	No		No	No	NR
Brain MRI/CT	MRI normal	Thin corpus callosum, low brainstem volume, variability in gyral pattern.	ND	ND		Ventricular dilatation, delayed myelination, peri-ventricular white matter lesion	MRI within normal limits	MRI normal
Growth parameters								
Microcephaly (≤3%)	Yes	Yes,< 1%	No	No		No	Yes,2%	No, 5%
Short stature (≤3%)	Yes	Yes	No	No		No	No	No
Overweight (BMI≥25)	No	No	No	No		No	No	No
Broad high forehead	Yes	Yes	Yes	Yes		No	No	NR
Other features		Mild joint laxity, subluxed hips, disordered sleep, feeding difficulties (g-tube dependency), laryngomalacia, micrognathia, abnormal palmar creases	Noonan facies, pes planus, hypospadias	Noonan facies, pes planus, hypospadias			Clinodactyly, nystagmus, abnormality soft palate	Cortical visual impairment

Abbreviations: %, centile; ASD, autism spectrum disorder; CT, computerised tomography scan; g-tube, gastrostomy tube; mod+, at least moderate severity;

MRI, magnetic resonance imaging; ND, not done; NA, not available; NR, not reported; NICU, neonatal intensive care unit;

VOUS, variant of uncertain significance. *Toe walking in absence of neurological signs of lower limb spasticity, therefore considered a behavioural manifestation.

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