De novo TNPO2 **variants are associated with developmental delays, neurologic deficits and dysmorphic features in humans and alter** ***TNPO2*** **activity in** ***Drosophila*****.**

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ABSTRACT

Transportin-2 (TNPO2) mediates multiple pathways including non-classical nucleocytoplasmic shuttling of >60 cargoes, including developmental and neuronal proteins. We identified fifteen individuals carrying *de novo* coding variants in *TNPO2* who presented with global developmental delay (GDD), dysmorphic features, ophthalmologic abnormalities, and neurological features. To assess the nature of these variants, functional studies were performed in *Drosophila*. We found that fly *dTnpo* (orthologous to *TNPO2*) is expressed in a subset of neurons. *dTnpo* is critical for neuronal maintenance and function as downregulating *dTnpo* in mature neurons using RNAi disrupts neuronal activity and survival. Altering the activity and expression of *dTnpo* using mutant alleles or RNAi causes developmental defects, including eye and wing deformities and lethality. These effects are dosage-dependent as more severe phenotypes are associated with stronger *dTnpo* loss. Interestingly, similar phenotypes are observed with *dTnpo* upregulation and ectopic expression of TNPO2, showing that loss and gain of Transportin activity causes developmental defects. Further, proband-associated variants can cause more or less severe developmental abnormalities compared to wild-type TNPO2 when ectopically expressed. The impact of the variants tested seems to correlate with their position within the protein. Specifically, those that fall within the RAN binding domain cause more severe toxicity and those in the acidic loop are less toxic. Variants within the cargo binding domain show tissue-dependent effects. In summary, *dTnpo* is an essential gene in flies during development and in neurons. Further, proband-associated *de novo* variants within *TNPO2* disrupt the function of the encoded protein. Hence, TNPO2-variants are causative for neurodevelopmental abnormalities.

KEYWORDS

Transportin; Drosophila; TNPO1; TNPO2; intellectual disability; global developmental delays; nucleocytoplasmic shuttling; rare disease; Karyopherin-β2b; Importin-3

INTRODUCTION

Genomic sequencing in combination with functional investigations in model organisms has led to the discovery of numerous novel Mendelian diseases[1,2]. Functional investigations may be particularly impactful when considering contributions of potential disease-associated variants that occur in genes encoding pleiotropic proteins[3,4], defined as proteins that function in a diverse number of unrelated pathways.

Here, we identified *Transportin-2* (*TNPO2* [MIM: 603002]; *Importin-3*; *Karyopherin-β2b*) as a disease associated gene. TNPO2 primarily mediates a non-classical nucleocytoplasmic shuttling pathway[5,6]. TNPO2 activity is dependent on the Ras-related nuclear protein (RAN) GTP/GDP gradient[7]. During nucleocytoplasmic shuttling, TNPO2 is bound by RAN-GDP at its N-terminus, promoting interactions with cytoplasmic protein cargoes at its C-terminus[5,6]. Subsequently, the RAN-GDP:TNPO2:cargo complex is shuttled into the nucleus via the nuclear pore complex (NPC). Conversion of RAN-GDP to RAN-GTP in the nucleus causes a conformational change in TNPO2's acidic loop – a flexible domain found between the RAN and cargo binding domains. This releases the cargo. RAN-GTP:TNPO2 is then shuttled back to the cytoplasm, destined to repeat the process.

TNPO2 is closely related to *Transportin-1* (*TNPO1* [MIM: 602901]*; Importin-2; Karyopherin-β2*)[5] and neither gene has been associated with a Mendelian disease. *TNPO2* is the less studied of the two as it was discovered later. Human TNPO2 and TNPO1 protein sequences are 84% identical and 92% similar[5]. Differences primarily occur in their flexible acidic loops and, to a lesser extent, their cargo-binding domains[8]. Current data support that TNPO2 and TNPO1 are functionally redundant[5]. Although the two genes are expressed ubiquitously, they differ in their expression levels in different tissues. Expression profiling data in mice demonstrated that *TNPO2* is more highly expressed in the brain than *TNPO1*[9]. These results are consistent with other mammalian datasets[5]. At the protein level, TNPO2 is more abundant in cultured neurons, astrocytes, and neural stem cells than TNPO1[10]. TNPO2 may also be more critical in muscles

as TNPO1 is not detected in cultured myoblasts[11]. TNPO2 is required during myoblast differentiation into myotubes[11].

More than 150 proteins are predicted to interact with TNPO1/2 based on high-throughput studies and over 60 proteins have been confirmed as TNPO1-cargoes[5,12,13]. Cargoes confirmed to be shuttled by TNPO2 include FUS (MIM: 137070)[14], HuR/ElavL1 (MIM: 603466)[8,11,15,16], hnRNPA1 (MIM: 164017)[8,17], and NF-κB Essential Modulator (NEMO [MIM: 300248])[18]. All of these are also TNPO1-cargoes. Recent high-throughput studies have detected rare proteins that uniquely interact with TNPO2[12,13] but direct investigations are needed to confirm them as TNPO2-specific cargoes.

The majority of TNPO1/2 cargoes carry a non-canonical nuclear-localization signal (NLS), a PY-NLS, defined as a C-terminal R/H/K-X_{2-5}PY motif[17]. However, a large number of cargoes do not have a PY-NLS and are simply described as being structurally disordered and having a hydrophobic or basic N-terminal sequence[5,19]. RNA-binding proteins and transcription factors needing import into the nucleus to regulate expression of a diverse number of genes are common targets of TNPO1/2[5]. Other nucleus-bound cargoes include histones, splicing factors, and ribosomal proteins[5,12,13]. TNPO1/2 also interacts with ciliary proteins[20–22], spindle assembly factors[23,24], and nucleoporins[23,25,26], shuttling these cargoes to the appropriate region of the cell for them to function. This means TNPO1/2 activity directly impacts ciliogenesis, mitotic spindle assembly, and nuclear envelope and pore assembly. Last, TNPO1/2 has been implicated in mechanisms that promote aging and neurodegenerative diseases[14,27–29].

Here we characterize a cohort that carry *de novo* variants within the *TNPO2* gene, finding that common features include developmental and neurological abnormalities. Using *Drosophila* to perform functional studies, we provide evidence that *de novo,* pathogenic variants in *TNPO2* are the genetic causes of individuals' symptoms.

MATERIALS AND METHODS

Recruitment and sequencing of individuals

Fifteen individuals were recruited through the Undiagnosed Diseases Network (UDN)[30] and GeneMatcher[31]. The procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national). Proper informed consent was obtained from family members for all probands in this study. All of the UDN work, including clinical and model organism work, and coordination for this publication, was performed under NIH IRB protocol 15-HG-0130.

Sequencing details for each proband can be found in **Data S1**. Briefly, trio (proband and both biological parents) whole exome sequencing (WES) was done in 14 of 15 cases as previously described[32–34]. Trio whole genome sequencing (WGS) used the Illumina Novaseq 6000 platform. Sequencing libraries were generated using the Truseq Nano DNA HT Sample Preparation Kit (Illumina, USA). Alignment of 150bp paired-end reads to the hg19 reference genome was performed using Burrows-Wheeler Aligner (BWA) software, before sorting with samtools and marking duplicates with Picard. Single nucleotide variants (SNVs) and small insertions/deletions (indels) were labelled using Genome Analysis Tool Kit (GATK v3.1), structural variants (SVs) were detected using DELLY (v0.7.3) software and copy number variants (CNVs) were detected using the control-FREEC (v9.9) tool. Following genomic variants detection, variants were annotated using ANNOVAR. Identification of genomic regions affected by each variant and possible changes in protein was performed using RefSeq and Gencode databases. The presence of the variants were assessed in dbSNP, GnomAD, 1000 Genomes Project, Exome Aggregation Consortium (ExAC), exome sequencing project (ESP) and Clinvar. Databases dbSNP, COSMIC, OMIM, GWAS Catalog and HGMD were used to find reported information of variants. SIFT, PolyPhen, MutationAssessor, LRT and CADD scores were used to predict the deleteriousness of mutations and GERP++ scores were used to access the conservation of mutations.

Drosophila husbandry and established fly lines

All fly lines were raised and maintained as described[35]. Publicly available fly lines are detailed in **Table S1** and were obtained from Vienna *Drosophila* Research Center (VDRC), Bloomington *Drosophila* Stock Center (BDSC), and Kyoto Stock Center (Kyoto). *Rh1-GAL4* on II (*w*; Rh1-GAL4;*), *elav-GAL4* on II (*y* w*; elav-GAL4*) and UAS control (*y w; PBac{UAS-empty}VK37/SM6a*) were published previously[36,37]. *da-GAL4*^{GS} (*w*; P{da-GSGAL4.T};*) was generously provided by H. Tricoire[38].

dTnpo mutant alleles and genomic rescue line

dTnpo^{Gly736Asp}*, Tnpo-RA* (NM_058020.4):*c.2207G>A (p.Gly736Asp)*, was identified in a forward genetic screen of *FRT80B* isogenized flies[39]. *dTnpo*$^{\Delta 11}$ is an imprecise excision line derived from *P{GawB}NP4408*[40] (Kyoto #104668). A *dTnpo* genomic rescue construct, *GR*^{dTnpo}, was cloned from the endogenous *dTnpo* gene using genomic DNA from isogenized *FRT80B* and inserted into the VK37 docking site using ϕC31 mediated transgenesis as described[41]. The *dTnpo CRIMIC (T2A-GAL4)* allele was designed as part of the Gene Disruption Project (construct CR92235) as described[42] using sgRNA 5' – CAAGCGTAATTTAAGAGTAATGG - 3'.

UAS-hTNPO2 lines

UAS-hTNPO2 lines were developed as described[35]. Q5 site directed mutagenesis (NEB # E00554S) was done on a pDONR223-hTNPO2 cDNA construct (NM_001136196.1; Horizon Discovery # OHS1770-202312693) to introduce a stop codon and variants. Primers are detailed in **Table S2**. LR clonase II (ThermoFisher # 11791020) was used to transfer the cDNA sequence to a pGW-attB-3xHA destination vector[43], creating pGW-hTNPO2 constructs. All clones were PCR and sequence confirmed. Sequencing primers included ones specific to the *hTNPO2* sequence (**Table S2**) and M13 primers. pGW-hTNPO2 constructs were inserted into

the VK37 docking site using ϕC31 mediated transgenesis[41]. Final genotype: *w*^{1118}*; PBac{UAS-hTNPO2}VK37/SM6a*.

GeneSwitch-driven transgene expression and lifespan

da-GAL4^{GS} and *elav-GAL4*^{GS} assays were performed as previously described[44] with the following changes. 1-2 day post-eclosion animals were placed onto 300μM for *elav-GAL4*^{GS} or 500μM for *da-GAL4*^{GS} RU486-containing food. RU486-food was prepared by mixing molten (60-65°C) food with 10mM RU486 (Sigma #M8046; prepared using 200pf ethanol) to the desired concentration at 2mL per vial. Molten food was solidified for 1-24h in a fume hood. For *elav-GAL4*^{GS} lifespan assays, female flies were maintained at 29°C. For *da-GAL4*^{GS} studies, female flies were maintained for 4 days on RU486 at 25°C.

Quantitative real-time polymerase chain reactions (qPCR)

qPCR was performed as previously described[44] with the following changes. The iScript gDNA Clear cDNA Synthesis Kit (BioRad #1725034), iTaq Universal SYBR Green Master Mix (BioRad #1725120) and a BioRad C1000 Touch Cycler were used. Multiple housekeeping genes (*RP49*, *RPS20*, and *Tubulin*) were included for normalizing the data. qPCR primers are described in **Table S2** and those for housekeeping genes were previously published[44].

Immunofluorescence (IF) and confocal microscopy

IF for L3 larval CNS and adult brains was performed as described[45,46]. Primary antibodies: anti-FasII (DSHB #7G10; 1:100), anti-Elav (DSHB #7E8A10; 1:500), anti-Repo (DSHB #8D12; 1:60), anti-mCherry (Genetex # GTX59788; 1:200; also targets RFP). Goat-derived secondary antibodies were used at 1:500 (Jackson ImmunoResearch Laboratories). A Leica Sp8x with lightning deconvolution was used for confocal microscopy. Images were taken with a 20x oil immersion Leica objective (HC PL APO 20x/0.75 IMM CORR CS2).

Western Immunoblots (WBs)

The BioRad Mini-PROTEAN Electrophoresis System was used with 4–20% Mini-PROTEAN TGX™ Precast Gel (BioRad #4561095), 1x Tris/Glycine/SDS running buffer, 1x Tris/Glycine transfer buffer with 10% methanol, and PVDF membrane. For lysates, whole frozen flies were homogenized as described[44,47] into 1x SDS sample buffer at 50µl per animal. For 1x SDS sample buffer, 60µL of β-mercaptoethanol was added to 1mL of diluted 6x SDS sample buffer [0.35M Tris-HCl (pH 6.8), 10% SDS, 30% glycerol, 30% β-mercaptoethanol, 1% Bromophenol Blue]. 10µL of lysate was loaded per lane. Membranes were stained and reprobed as described[44,47]. Antibodies: anti-hTNPO1/2[A11] (1:1000, Santa Cruz #sc-365179), anti-mouse-HRP (1:5000), and anti-α-Tubulin[11H10]-HRP (1:2000, Cell Signaling #9099). HRP activity was measured using SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Sci #34577) and a BioRad Chemidoc MP Imaging System.

RESULTS

Coding variants in TNPO2 are associated with global developmental delay, dysmorphic features, ophthalmologic abnormalities, and neurological features.

Fifteen individuals who primarily presented with feeding difficulties and developmental delays during infancy or in early childhood were evaluated clinically by their providers in the respective institution (**Data S1**). Trio (proband and both biological parents) sequencing, primarily whole exome sequencing (WES), was performed by these clinical sites and results showed that these individuals carry a potentially pathogenic, heterozygous coding-variant in *TNPO2* (NM_001136196.1) (**Table 1**; extended data in **Data S1**). Based on the presence of this variant, individuals were recruited to this study through the Undiagnosed Diseases Network (UDN)[44] and GeneMatcher[45], independent of their respective clinical features.

All variants are *de novo* except the one in proband 13, whose mother was low-level mosaic. The variant is in 1% of NGS reads in the mother by WES. To learn more about *TNPO2* and potential impact of these variants, we used information accumulated into the Model organism Aggregated Resources or Rare Variant ExpLoration (MARRVEL) tool, v2[48]. MARRVEL is a valuable resource that brings together multiple sources of information for the investigation of human and model organism based disease research. Here, we found that *TNPO2* is highly constrained, having a missense constraint (misZ) score[49] of 5.88 (observed/expected (o/e) = 0.28) and a probability of loss-of-function intolerance (pLI) score[49,50] of 1.00 (o/e = 0.04) based on gnomAD (genome Aggregation Database), v2.1.1[51]. Twelve probands carry single nucleotide variants (SNVs) in *TNPO2* that are predicted to be deleterious using combined annotation dependent depletion (CADD) scores (phred >20), v1.4[52]. Proband 5 carries a mosaic, in-frame deletion of p.Lys152del (16% by Sanger, 21% by WGS of reads, DNA from blood). Proband 9 carries a deletion-insertion of p.Lys491_Arg492delinsGlnTrp. Proband 14 carries an in-frame deletion of p.Ala649_Leu652, removing four codons. None of these variants are found in genetic databases containing control populations, including information in gnomAD[51].

To evaluate common features among probands, information was extracted from chart review as well as clinic visits at the respective institutions. We found that all probands present with global developmental delay (GDD), with either slow or plateaued developmental progress (**Tables 1** and **S1**). Probands 2, 4 and 13 show regression of milestones, mostly transiently. All probands have delayed speech, with expressive language more severely impacted compared with receptive speech. Four individuals are nonverbal (+++) including proband 7 at age10 years and proband 12 at age 20 years. Motor impairments appear to be comparatively less severe in our cohort compared with speech delays, although probands 5, 7 and 12 are non-ambulatory (+++). Intellectual disability (ID) was assessed and found in nine probands, ranging from mild (+) to severe (+++). ID is also suspected in another three individuals.

Behavioral deficits are observed in ten of fourteen probands with variable presentation (**Tables 1** and **S1**). The most common neuropsychiatric concerns are inattention and autistic behaviors. Proband 12 is severely delayed so behavioral analysis was not done.

Gastrointestinal (GI) features appear to be shared within the cohort, impacting eleven of fifteen probands (**Tables 1** and **S1**). The most common features include neonatal feeding difficulties and poor weight gain.

No single craniofacial dysmorphism is reported across the cohort, although dysmorphic features are noted in eleven of fifteen individuals (**Fig. 1; Tables 1** and **S1**). The most common abnormalities include a broad or high nasal bridge, retrognathia, and a shortened philtrum. Skull and facial features include dolichocephaly, bitemporal narrowing or narrow face/high arched palate in four probands, and microcephaly (defined as less than –2 standard deviations; SD) in five probands. Noticeably, dysmorphisms surrounding the eye area are observed in five probands although presentation varies. This includes deep-set eyes and palpebral fissure length, spacing or slant irregularities. Dysmorphic ears are also noted in six probands.

Ophthalmologic abnormalities are reported within the cohort and impact ten of fifteen individuals (**Tables 1** and **S1**). Strabismus is observed in seven probands. Saccadic and rapid eye movements are noted in three individuals, resolving in proband 5 by age 23 months. Myopia, hyperopia, or astigmatism are described in four probands.

Muscle tone abnormalities are described in eleven of fifteen individuals, primarily hypotonia. Interestingly, probands 6 and 10 show signs of both hypertonia and hypotonia. In addition, movement and neurological disorders, primarily tremors and ataxia, were seen in six of fifteen probands.

Neurologic impairments are detected in some probands (**Tables 1** and **S1**). Six of the fifteen individuals had seizures starting between 1 to 2.5 years-of-age. Initial presentation in five of these individuals was febrile-induced and in four of five cases, individuals developed non-febrile seizures. Electroencephalograms (EEGs) were abnormal in three of ten probands assessed,

with proband 7 showing severe abnormalities consistent with epileptic encephalopathy. Magnetic resonance imaging (MRI) of the brain was done on thirteen probands and cerebellar hypoplasia or dysplasia were seen in three probands. *Tnpo2* is highly expressed in this region in mice[53]. Other findings include white matter loss, mild ventricular dilation, hypoplastic caudate nuclei, thin corpus callosum as well as minor anomalies such as cavum septum pellucidum, enlarged Virchow Robin spaces, and borderline delay in myelination.

Other, less common features for individuals are also observed. This includes renal abnormalities (bilateral pyeloureteral junction stenosis requiring surgery at age 3 months in proband 4, left kidney agenesis in proband 8, and kidney stones in proband 12), nipple abnormalities, cardiac abnormalities (patent ductus arteriosus requiring transcatheter closure in proband 7, mild dilation of the aortic root in proband 12), finger anomalies, hip dysplasia, (kypho)scoliosis, and pes planus (**Data S1**).

Six of fifteen probands carry additional heterozygous, *de novo* genomic alterations which were not the primary candidate for further investigation (detailed in **Note S1**). Briefly, probands 6, 8, and 15 carry SNVs of uncertain significance (VUS) in *Rabankyrin-5* (*ANKFY1* [MIM: 607927]*;* no disease association), *Armadillo repeat containing 9* (*ARMC9* [MIM: 617612]*;* associated with autosomal recessive Joubert Syndrome 30 [MIM: 617622]), and *α-Internexin* (*INA* [MIM: 605338]; no disease association), respectively. Importantly, these genes are less constrained than *TNPO2* (see **Note S1**). Proband 5 carries two VUS that are not predicted to be pathogenic based on CADD and other information (see **Note S1**), including a SNV in *Cut-like Homeobox 2* (*CUX2* [MIM: 610648]; associated with autosomal dominant developmental and epileptic encephalopathy 67 [MIM: 618141]) and a duplication of 12q13.13. This individual also has a deletion-insertion in *SET binding protein 1* (*SETBP1* [MIM: 611060]) that occurs considerably further down in the gene from known pathogenic variants associated with mental retardation, autosomal dominant 29[54] and has no suggestive features for Schinzel-Giedion syndrome[55] (MIM: 269150; autosomal dominant). Proband 10 carries multiple VUS (see

Note S1), most notably a 522Kb gain in 1q21.1. No impacted genes were thought to explain the individual's features. Proband 14 carries a truncating SNV in the highly constrained gene *Phosphodiesterase-4D* (*PDE4D* [MIM: 600129]) and is diagnosed with acrodysostosis 2[56] (ACRDYS2 [MIM: 614613]).

In summary, fifteen individuals were identified who carry potential disease-causing variants in *TNPO2*. All individuals present with global developmental delays. Speech abilities and intelligence are typically more impaired than motor abilities. Other common features between probands include variable dysmorphic features, ophthalmologic abnormalities (primarily strabismus), muscle tone abnormalities (primarily hypotonia), movement/neurological disorders, and neurological features.

Drosophila Tnpo is orthologous to human TNPO2 and most proband variants affect evolutionarily conserved residues.

To investigate if the *TNPO2* variants identified in our cohort underlie individuals' features, we utilized the model organism, *Drosophila melanogaster*. The fly orthologue to human *TNPO2* (*hTNPO2*) is *Drosophila Tnpo* (*dTnpo*) and the encoded proteins from these two genes shuttle the same cargoes into the nucleus[57–61]. The amino acid sequences encoded by these two genes are 71% identical and 84% similar (**Fig. 2A**). The DRSC Integrative Ortholog Prediction Tool (DIOPT, v7.1)[62] score between these genes is 13 of 16, giving strong confidence that *dTnpo* is indeed orthologous to *hTNPO2*. The sequences of the RAN binding and cargo binding domains are more conserved than that of the acidic loop. Specifically, sequences encoding the acidic loops are 59% identical and 70% similar compared to the RAN binding domain (69% identical, 84% similar) and cargo binding domain (74% identical, 85% similar)[8]. dTnpo is also orthologous to human TNPO1 (DIOPT score = 13/16), so one fly gene corresponds to two human genes.

Fourteen of fifteen variants found within our cohort occur at conserved amino acids between hTNPO2 and dTnpo (**Fig. 2A, red**). Five variants are within the RAN binding domain. Two

variants are at the same position within the acidic loop. Seven variants localize to the cargo binding domain. The p.Lys118Asn variant associated with proband 4 is not at a conserved amino acid (**Fig. 2A, orange**) and the amino acid within the fly protein is an asparagine (Asn; N). This variant is at a conserved amino acid in vertebrate models (see data in MARRVEL).

Developmental loss of dTnpo causes lethality and morphologic defects.

To gain an understanding of whether *hTNPO2* may be essential during development, we assessed phenotypes associated with *dTnpo* loss in the fly. For this purpose, three *dTnpo* loss-of-function (LOF) mutant alleles were generated using different strategies (**Fig. 2B**). First, we previously identified $dTnpo^{Gly736Asp}$ in a genetic screen[39]. Second, a truncated *dTnpo* mutant, $dTnpo^{\Delta 11}$*,* was generated by an imprecise excision of a P-element. Last, a *CRISPR-Mediated Integration Cassette (CRIMIC)* allele was created by insertion of a *Splice Acceptor-T2A-GAL4-polyA* sequence into a shared intron of all *dTnpo* transcripts, effectively disrupting the gene's expression by creating a truncated mRNA[42]. We also obtained two available *UAS-RNAi* fly lines designed to target *dTnpo*[63,64] (**Fig. 2B**). These RNAi lines effectively reduce expression of *dTnpo* based on qPCR. *dTnpo* RNAi-1 causes an 81 ± 0.05% reduction and *dTnpo* RNAi-2 (previously used in[60]) causes a 58 ± 0.16% reduction of *dTnpo* mRNA compared to control RNAi expressing animals (**Fig. S1A**).

The three *dTnpo* mutant alleles are homozygous lethal and no obvious phenotypes are observed in heterozygous animals. Notably, homozygous mutant animals do not survive beyond larval stages of development, shown in **Fig. 2C.** In rare cases, escaper puparia could be observed in $dTnpo^{Gly736Asp}$ cultures and, less commonly, in $dTnpo^{\Delta 11}$ cultures. Based on a complementation test with a deletion line that lacks *dTnpo*, *Df(3L)Exel8101*, we conclude that $dTnpo^{\Delta 11}$ is the most severe LOF allele, causing lethality at larval stage 2 (L2). $dTnpo^{Gly736Asp}$ behaves as a hypomorph based on complementation failure with *Df(3L)Exel8101,* causing death in larval stage 3 (L3). Finally, the *dTnpo CRIMIC* allele also behaves as a hypomorph,

causing lethality between L2 and L3. The *dTnpo*$^{\Delta 11}$ and *dTnpo*^{Gly736Asp} alleles are rescuable by a genomic rescue line, *GR*^{dTnpo}, which carries an independent copy of *dTnpo*. Consistent with these data, ubiquitous expression of the strong *UAS-dTnpo RNAi-1* using *da-GAL4* causes lethality at L2, similar to *dTnpo*$^{\Delta 11}$ mutants (**Fig. S1B**). Further, *da-GAL4* driven expression of the weaker *UAS-dTnpo RNAi-2* causes lethality at L3, similar to *dTnpo*^{Gly736Asp}. Overall, these data show that *dTnpo* is essential during fly development.

Since *hTNPO2* is likely required in multiple tissues and probands with *hTNPO2* coding variants have diverse features, we assessed if *dTnpo* loss impacts different tissues. Given that the majority of our cohort have ophthalmologic abnormalities, we first focused on the fly eye. The formation of this tissue is well studied and the developmental pathways required for proper eye formation are conserved[65]. The mutant alleles *dTnpo*^{Gly736Asp} and *dTnpo*$^{\Delta 11}$ were recombined onto *FRT80B*-chromosomes. Using the FRT/FLP system[66], we crossed these flies to *ey-FLP GMR-lacZ;; RpS174 w+ FRT80B* to create mosaic eyes that include either homozygous mutant clonal tissue (white) or wild-type clonal tissue (red) (**Fig. 2D**). Compared to *FRT80B* controls, *dTnpo*^{Gly736Asp} *FRT80B* causes eye deformities, including disorganized ommatidia consistent with a rough eye phenotype and smaller eyes. Interestingly, no homozygous mutant tissue is seen in animals carrying the stronger mutation, *dTnpo*$^{\Delta 11}$ *FRT80B*, demonstrating that *dTnpo* is essential for eye development. Expression of *dTnpo* RNAi in the developing fly eye using *ey-GAL4* shows consistent results, with the stronger *UAS-dTnpo RNAi-1* causing developmental lethality and the weaker *UAS-dTnpo RNAi-2* causing a rough eye phenotype and small eyes (**Fig. S1D**). Thus, effects of *dTnpo* loss on eye development seem to be dosage dependent. Interestingly, expressing *dTnpo* RNAi with *GMR-GAL4*, which expresses later in eye development, did not cause significant alterations to the external fly eye (**Fig. S1E**). These data argue that *dTnpo* is required during early eye imaginal disc development but do not rule out a requirement at later stages.

We next tested for *dTnpo* requirement during fly wing development, also a well-studied tissue that involves conserved signaling pathways for proper formation[67,68]. We created mosaic tissue in the wing disc of *dTnpo*^{Gly736Asp} *FRT80B* larvae using *Ubx-FLP;; Ubi-GFP FRT80B*. Interestingly, wing notch phenotypes and large blisters can be observed in *dTnpo*^{Gly736Asp} mutant animals (**Fig. 2E**). Taking an alternative approach, we used *nub-GAL4* to express *UAS-dTnpo RNAi* in the developing wing disc and partially in the thorax. The stronger RNAi-1 causes lethality, consistent with *dTnpo* being required for development. The weaker RNAi-2 causes severe defects in wing morphology with hardly any wing forming (**Fig. S1C**). Hence, *dTnpo* is required for wing development.

In sum, we found that *dTnpo* is required in multiple fly tissues for proper development using *dTnpo* LOF reagents. Interestingly, *dTnpo* loss was dosage-dependent with the stronger mutant allele, *dTnpo*$^{\Delta 11}$, and the stronger *dTnpo* RNAi, causing more severe defects than other hypomorphic reagents during eye and wing development.

dTnpo is expressed primarily in neurons of the fly CNS.

Given that the majority of the *hTNPO2* cohort have features commonly associated with neurologic deficits and *TNPO2* is highly expressed in the mammalian brain[9,10], we explored the importance of *dTnpo* in this tissue. First, we defined *dTnpo*'s expression pattern in the L3 larval central nervous system (CNS) and adult fly brain (**Fig. 3**). The *dTnpo CRIMIC* allele (see **Fig. 2B**) carries a *T2A-GAL4* sequence that expresses a GAL4 transcription factor under control of *dTnpo's* regulatory elements[42]. This GAL4 can drive expression of any *UAS-transgene* in the same spatial and temporal pattern as *dTnpo*[69]. Thus, we used the *dTnpo CRIMIC* allele to express *UAS-mCherry.NLS* (mCherry fluorescent protein localized to the nucleus) (**Fig. 3A-L**). In larvae, mCherry (*dTnpo*) staining is most common in the central brain, including the cell bodies of mushroom body (MB) neurons, and ventral nerve cord (VNC; corresponding to the mammalian spinal cord) (**Fig. 3A and G**); a schematic of the larval CNS is shown in **Fig. 3S** for

reference. These are areas that harbor a high density of active neurons at this stage[70]. In adults, mCherry (*dTnpo*) staining shows the highest density in the optic lobe, MB cell bodies, and the central complex (**Fig. 3D and J**); a schematic of the adult brain is shown in **Fig. 3T** for reference.

To define localization to specific cell types, tissue were counterstained with Elav (predominantly marks neurons) or Repo (predominantly marks glia except midline glia)[71–73]. In both the larval CNS (**Fig. 3B-C)** and adult brain (**Fig. 3E-F)**, not all Elav-positive cells stain positive for mCherry (*dTnpo*) in whole-mount, Z-stacked images, supporting that *dTnpo* is expressed in a subset of neurons. These findings were consistent when using single-slice images of regions that show high mCherry staining in both the larval CNS (**Fig. 3A'-C'**) and adult brain (**Fig. 3D'-F'**). In whole mount with Z-stacked images, there is no obvious overlap with glia and cells expressing mCherry (*dTnpo*) in larvae (**Fig. 3H-I**) and adults (**Fig. 3K-L**). However, in single-slice images of larval CNS (**Fig. 3G'-I'**) and adult brains (**Fig. 3J'-L'**) some Repo-positive cells show overlap with mCherry-positive cells, arguing that a small subset of glia express *dTnpo*.

The MB is of interest as this is the primary learning and memory center in *Drosophila*[74] and the individuals in our cohort present with intellectual disability. To confirm *dTnpo* expression in these cells, *UAS-mCD8::RFP* (RFP fluorescent protein localized to the membrane) was expressed using the *dTnpo CRIMIC* allele and tissue were counterstained with an established MB marker, FasII[75], in larvae (**Fig. 3M-O**) and adults (**Fig. 3P-R**). Indeed, we see consistent overlap between RFP (*dTnpo*) and FasII signal, supporting that *dTnpo* is expressed in these neurons.

In sum, we found that *dTnpo* is highly expressed in a subset of neurons, including those that mediate associative learning, in the larval CNS and adult brain.

dTnpo is required for neuron function and maintenance.

We next examined if *dTnpo* was essential in fly neurons. We first assessed if *dTnpo* was required during neural development. When we express *UAS-dTnpo RNAi-1* in neuroblasts (neural stem cells) using *insc-GAL4*, no significant reductions in L3 larval CNS size are seen (**Fig. S2**). In contrast, expressing *UAS-dTnpo RNAi-1* using the pan-neuronal driver, *elav-GAL4,* is lethal (**Fig. S1F**).

To avoid developmental lethality caused by expressing *UAS-dTnpo RNAi-1* with *elav-GAL4*, we utilized a drug, RU486, inducible version of this neuronal driver, *elav-GAL4[GeneSwitch]* (*elav-GAL4*^{GS})[76] to express the *dTnpo* RNAi-1. 1-2d flies were transferred onto RU486-containing food, thus avoiding RNAi expression prior to adulthood. These animals were maintained on RU486 and survival curves were calculated for animals expressing *UAS-dTnpo RNAi-1* compared to animals expressing *UAS-control (Luciferase) RNAi*. Interestingly, there is a significant decrease in survival when *dTnpo* is downregulated using RNAi expression in the adult fly neurons (**Fig. 4A**). 50% of *UAS-dTnpo RNAi-1* expressing animals die by 22d compared to 32d for control RNAi expressing animals. The max survival is also decreased by 20d with 100% of *dTnpo* RNAi-1 animals dying by 28d, compared to 48d for control RNAi animals.

To examine if *dTnpo* is required for neuronal activity, we performed electroretinograms (ERGs) on *UAS-dTnpo RNAi-1* and *UAS-control RNAi* expressing animals (**Fig. 4B-F**). ERGs are an established method for measuring neuron dysfunction in the synaptic circuit that makes up the fly optic system as these allow for the quantification of light coincident receptor potentials (LCRP) and ON/OFF transients[77–79]. LCRP amplitudes measure the phototransduction pathway that is dependent on light exposure[77,78]. ON/OFF transients measure synaptic transmission between photoreceptor neurons and post-synaptic neurons in the lamina[77,78]. At 7d, the downregulation of *dTnpo* by expressing RNAi in mature photoreceptor neurons using *Rh1-GAL4* causes significant changes to the ON and OFF transient amplitudes (**Fig. 4D-E**), indicating a loss of synaptic activity. LCRP defects are also observed in *dTnpo* RNAi-1 expressing animals

based on reductions in depolarization amplitude (**Fig. 4F**). This impact seems to become stronger with age as there is a reduction of 24% in LCRP at 7d when compared to control RNAi expressing flies. This reduction is more robust by 14d and 22d at 42% and 44%, respectively.

In sum, *dTnpo* expression in neurons was found to be essential for animal survival. Further, neuronal function in the fly eye is disrupted by *dTnpo* loss, supporting that *dTnpo* is required in mature neurons.

Upregulation of dTnpo causes similar phenotypes to dTnpo LOF mutants.

Thus far, we found that phenotypes associated with *dTnpo* loss are dosage-dependent. We therefore considered if *dTnpo* over-expression could also be detrimental. We obtained a fly line, *UAS::dTnpo*^{GS11030}, that contains a P-element insertion with a *UAS* element upstream of the *dTnpo* gene[80]. This allows us to upregulate *dTnpo* under control of the *GAL4/UAS* system[69] by a 25 ± 8.1 fold increase in *dTnpo* mRNA levels (**Fig. 5A**). Interestingly, upregulation of *dTnpo* using the ubiquitous driver, *da-GAL4,* causes lethality after pupariation (**Fig. 5B**). Further, upregulation of *dTnpo* in both early (*ey-GAL4*) and late (*GMR-GAL4*) eye development causes rough eye phenotypes and reduced eye size (**Fig. 5B-C**). *dTnpo* upregulation in the wing using *nub-GAL4* causes wing notching and large blisters in 100% of animals (**Fig. 5D**). In sum, we see that upregulating *dTnpo* causes similar phenotypes as *dTnpo* loss (see **Fig. 2** and **S1**).

Ectopic expression of human TNPO2 in flies causes toxicity in vivo.

We next aimed to define if proband-associated variants in *hTNPO2* could alter the function of the encoded protein *in vivo*. For this purpose, we established a series of *UAS-hTNPO2* fly lines expressing wild-type (WT; reference) or variant human *TNPO2* cDNA (NM_001136196.1) under control of the *GAL4/UAS* system. We selected six of the fourteen variants that are at conserved amino acids for analysis, two from each protein domain. This included p.Gln28Arg and p.Asp156Asn, p.Trp370Arg, p.Trp370Cys, p.Ala546Val and p.Trp727Cys. We confirmed

that these lines properly express the *UAS-hTNPO2* transgenes at comparable levels using western immunoblots and an antibody specific to human TNPO1/2 (**Fig. 6A**). We expressed *UAS* transgenes in 1-2d adult animals using the drug-inducible, ubiquitous driver, *da-GAL4*^{GS}, to avoid any toxicity during development.

Previously, we had found that animals trans-heterozygous for the hypomorph alleles, *dTnpo*^{Gly736Asp} and *dTnpo CRIMIC*, did not survive past larval stage 3 (see **Fig. 2C**). Thus, we examined if expression of *hTNPO2* could rescue this phenotype. Using the *dTnpo CRIMIC (T2A-GAL4)* allele, we expressed *UAS-hTNPO2:WT*^{HA} (wild-type *hTNPO2* cDNA with a 3' 3xHA-tag) or control *UAS-(empty)* in these *dTnpo* trans-heterozygous hypomorph animals. No rescue is observed with the expression of *hTNPO2* cDNA in these mutant animals (**Fig. S3A**). We noted that *hTNPO2:WT* expression causes death earlier, at L2, rather than L3 when compared to *UAS-(empty)* control expressing flies, demonstrating that the expression of *hTNPO2:WT* increased, rather than reduced, toxicity in these *dTnpo* mutant flies. We also tested if five of the variants found in our cohort could rescue lethality in *dTnpo* trans-heterozygous animals. Interestingly, the p.Trp370Cys and p.Ala546Val variants result in death at L3 rather than L2 when compared to *hTNPO2:WT*. The other variants tested – p.Gln28Arg, p.Asp156Asn, p.Trp727Cys – caused lethality at L2 like *hTNPO2:WT*.

We next considered if the lack of rescue seen with expression of *hTNPO2* in *dTnpo* trans-heterozygous hypomorph animals is due to the overexpression of *hTNPO2* in flies being toxic. This is because we found that robust upregulation of *dTnpo* was toxic (see **Fig. 5**) and the expression of *UAS-hTNPO2* by the *dTnpo CRIMIC* allele will result in an overexpression of *hTNPO2*[42], albeit likely at significantly lower levels than that caused by the *UAS::dTnpo*^{GS11030} allele[69]. Thus, we would have two sources of toxicity in rescue experiments, that from the mutations in *dTnpo* and that from overexpressing *hTNPO2*. To test this hypothesis, Mendelian ratios were calculated for progeny from crosses between *da-GAL4* and *UAS* fly lines, including *UAS-hTNPO2* lines and the control *UAS-(empty)* line (**Fig. 6B**). As expected, 58 ± 2.1% of

control progeny carry the *UAS* transgene. In contrast, only 28 ± 6.4% of progeny from *UAS-hTNPO2:WT*^{HA} crosses carry the *UAS* transgene, showing that significant toxicity occurs during development (**Fig. 6B**). The presence of the HA-tag on the *hTNPO2:WT* transgene does not alter this effect (**Fig. S3B**). Overall, these data demonstrate that ubiquitous, ectopic expression of *hTNPO2* is toxic.

We also investigated if proband-associated variants could induce the same toxicity as wild-type *hTNPO2*. Notably, *p.Gln28Arg* and *p.Asp156Asn* are more toxic than *hTNPO2:WT* (**Fig. 6B**). Further, variants *p.Trp370Arg*, *p.Trp370Cys*, and *p.Ala546Val* cause similar toxicity compared to that caused by *hTNPO2:WT* expression (**Fig. 6B**). In contrast, the *p.Trp727Cys* variant is significantly less toxic than *hTNPO2:WT*, producing 50 ± 8.2% of *UAS* carrying progeny (**Fig. 6B**).

In summary, ectopic expression of *hTNPO2* in flies causes toxicity consistent with phenotypes observed when upregulating *dTnpo* (see **Fig. 5**). As three of six proband-associated variants tested caused significant differences in the amount of toxicity than that caused by wild-type TNPO2, these data suggest that these variants alter the function of the hTNPO2 protein. Specifically, p.Gln28Arg and p.Asp156Asn may cause gain-of-function (GOF) effects and p.Trp727Cys may cause LOF effects.

Toxicity caused by variants in the fly eye differ from that of wild-type hTNPO2.

Next, we assessed if ectopically expressing wild-type and variant *hTNPO2* in the fly eye can cause morphologic disruptions similar to wild-type *dTnpo* upregulation (see **Fig. 5**). Using *ey-GAL4,* expression of *hTNPO2:WT* causes a smaller eye and a rough eye phenotype compared to animals expressing a *UAS* control (**Fig. 6C**). Expression of the *p.Trp727Cys* variant leads to eyes more similar to controls than *hTNPO2:WT* (**Fig. 6C**), consistent with it being less toxic than *hTNPO2:WT* during animal development (see **Fig. 6B**). Interestingly, variants *p.Trp370Arg* and *p.Trp370Cys* are also less toxic than *hTNPO2:WT*, suggesting that during early eye

development these variants act as LOF-variants. Expression of variants *p.Gln28Arg*, *p.Asp156Asn*, and *p.Ala546Val* cause similar eye phenotypes as *hTNPO2:WT*.

To further explore the impacts of variants at later stages of the developing eye than those affected by *ey-GAL4*, we expressed *UAS* transgenes using *GMR-GAL4*. Ectopic expression of *hTNPO2:WT* causes a moderate rough-eye phenotype and smaller eyes compared to animals expressing *UAS* control (**Fig. 6D**). Consistent with previous data using *da-GAL4* (see **Fig. 6B**), expression of *p.Gln28Arg* and *p.Asp156Asn* cause more toxicity than *hTNPO2:WT* expression (**Fig. 6D**). Specifically, *p.Gln28Arg* causes a more robust rough eye phenotype and smaller eyes and *p.Asp156Asn* causes developmental lethality. *GMR-GAL4* is expressed at low levels in the larval brain[81,82] and has been reported to cause lethality in extremely toxic situations[44,83]. In contrast, eye phenotypes caused by expression of *p.Trp370Arg*, *p.Trp370Cys* and *p.Ala546Val* are similar to *hTNPO2:WT* (**Fig. 6D**). In turn, expressing *p.Trp727Cys* causes a slightly more robust rough eye phenotype compared to *hTNPO2:WT* expressing animals.

In sum, ectopic expression of wild-type *hTNPO2* in the fly eye causes morphologic defects. Interestingly, these defects are different when comparing animals expressing proband-associated variants versus *hTNPO2:WT*. The specific effects of each variant are dependent on the developmental stage during which the transgenes are expressed in the fly eye, fitting with *TNPO2* encoding a pleiotropic protein that may play different roles at different stages of development.

Toxicity caused by variants in the developing wing differ from that of wild-type hTNPO2.

Thus far, we defined differences between toxicity induced by ectopically expressing wild-type and variant *hTNPO2* by expressing them ubiquitously during development or in the developing fly eye (see **Fig. 6**). Given that toxicity in response to expressing these proteins may differ depending on the tissue, we further analyzed impacts in the fly wing using *nub-GAL4*. When *UAS-hTNPO2:WT^{HA}* was ectopically expressed in the developing wing, progeny had

blister phenotypes, gain-of-vein phenotypes and the wings were smaller than control *UAS* wings (**Fig. 7A**). *hTNPO2:WT* phenotypes are 50 ± 8.5% penetrant, allowing us to quantitatively analyze the effects of proband-associated variants in this tissue (**Fig. 7B**). Consistent with previous data, expression of *p.Gln28Arg* and *p.Asp156Asn* cause more severe wing phenotypes than *hTNPO2:WT* expressing animals (**Fig. 7A**). In addition to blisters and gain-of-vein phenotypes, wings from *p.Gln28Arg* and, particularly, *p.Asp156Asn*, commonly have notch phenotypes, are smaller, and have more disruptions to wing inflation than *hTNPO2:WT* animals. Further, blister and notch phenotypes are significantly more penetrant with these variants, at 99.6 ± 0.2% and 98.8 ± 0.5%, respectively (**Fig. 7B**). In contrast, animals expressing variants *p.Trp370Arg*, *p.Trp370Cys*, and *p.Ala546Val* rarely have blister phenotypes (**Fig. 7A**) with a concomitant and significant reduction in phenotype penetrance compared to *hTNPO2:WT* expressing animals (**Fig. 7B**). Interestingly, animals expressing *p.Ala546Val*, which had minimal effects in the developing animal and eye (see **Fig. 6**), showed few defects and phenotype penetrance was significantly lower than that for *hTNPO2:WT* expressing animals at 0.8 ± 0.5%. Last, expression of *p.Trp727Cys*, which had variable effects in other tissues, causes a more severe gain-of-vein phenotype compared to *hTNPO2:WT* expressing animals (**Fig. 7A**). However, penetrance of the blister and notch phenotypes is similar between animals expressing *p.Trp727Cys* and *hTNPO2:WT* (**Fig. 7B**).

In sum, ectopic expression of *hTNPO2* in the developing fly wing causes morphologic defects that are altered by proband-associated variants. Concomitantly, phenotype penetrance is significantly different for most variants.

DISCUSSION

We identified fifteen individuals who carry pathogenic coding variants in the pleiotropic protein, TNPO2. Probands uniformly present with global developmental delay (GDD), including speech/motor deficits and intellectual disability. The majority also have behavioral deficits,

feeding difficulties, dysmorphic features, strabismus, and muscle tone abnormalities (primarily hypotonia). Movement and neurological disorders (e.g. tremors, ataxia) and neurological features, including seizures and abnormal MRIs, are also seen. Using *Drosophila*, we found that *Transportin* (*dTnpo*) is an essential gene during animal development and required for proper eye and wing formation. Malformations caused by decreasing *dTnpo* activity are dosage-dependent with greater reductions in *dTnpo* activity causing more severe defects. Interestingly, upregulation of *dTnpo* causes similar defects as *dTnpo* loss. We further found that *dTnpo* is required in the fly nervous system. It is expressed mostly in neurons and abundant in mushroom body (MB) neurons. Downregulating *dTnpo* in mature neurons disrupts their function and reduces animal survival. Ectopic expression of wild-type, human *TNPO2* causes similar phenotypes in the fly as gain and loss of *dTnpo*, suggesting that its function is evolutionarily conserved. Interestingly, ectopic expression of *UAS-hTNPO2* transgenes carrying proband-associated variants cause different levels of toxicity compared to animals expressing wild-type *UAS-hTNPO2*, supporting that these variants disrupt the encoded protein's normal activity *in vivo*. Impacts seem to vary based on the variant's location within the protein (discussed below). In conclusion, these data demonstrate that *de novo* coding variants within *TNPO2* can alter the function of the encoded, essential protein and are associated with developmental phenotypes in individuals.

Potential roles of TNPO2 variants in disease.

While *de novo* variants in *TNPO2* had been recorded in individuals with intellectual disability, previous studies did not find that *TNPO2* variants were a significant cause of GDD in large datasets that included hundreds to thousands of cases[84–87]. Our independent identification of fifteen individuals with GDD that carry pathogenic *TNPO2* variants and functional studies using *Drosophila* provide strong evidence of the important role this gene plays in human

development and in the nervous system. Together, these data demonstrate that while *TNPO2* variants are associated with GDD, this is a rare cause of disabilities.

Our fly data demonstrate that all variants tested can alter the anticipated function of hTNPO2 *in vivo* (see **Figs. 6-7**). These data suggest that the impact of a variant depends on its position within the protein in addition to the tissue and developmental stage during which the transgenes are expressed. To better understand how these variants compare to wild-type TNPO2, we compiled a summary of our fly data that used ectopic expression of *TNPO2* cDNA (**Fig. 7C**; extended data in **Fig. S3C**). Interestingly, expression of variants that fall within the RAN binding domain were significantly more toxic than hTNPO2:WT in almost all conditions. These data suggest these are GOF variants. In contrast, expression of variants that fall within the acidic loop of the protein tended to be less toxic than *hTNPO2:WT* expression, suggesting they are LOF variants. As these two variants fall at the same amino acid in the protein, future studies are needed to test additional variants from within this domain. Interestingly, we also observed that expression of variants in the cargo binding domain of hTNPO2 have more variable effects when compared to *hTNPO2:WT* expression. Generally, these have reduced toxicity compared to wild-type *hTNPO2*, but the tissue assayed seems more critical in defining their impact. This fits with the known function of this domain as cargoes are likely to differ between tissues and ages. Specifically, in our fly data *UAS-hTNPO2:p.Ala546Val* (proband 11's variant) has no impact when ubiquitously expressed or when expressed in the fly eye compared to *UAS-hTNPO2:WT*^{HA}. However, expression of this variant is significantly less toxic than *hTNPO2:WT* in the fly wing. Consistent with these data, proband 11 shows fewer phenotypes than other persons within our cohort (see **Table 1, Data S1,** and **Fig. 1**). Thus, this variant may be tissue-specific. In addition to *p.Ala546Val*, the current fly data argue that the impact of *p.Trp727Cys* is also tissue dependent. Specifically, expression of this variant is significantly less toxic than wild-type *TNPO2* with *da-GAL4* (ubiquitous) and *ey-GAL4* (early eye formation) and only mildly more toxic than wild-type *TNPO2* with *GMR-GAL4* (late eye formation) and *nub-*

GAL4 (wing formation). In sum, these data suggest that it is more of a LOF-variant than a GOF-variant while mechanistic studies are needed in each tissue to strengthen these results. Interestingly, disease presentation in proband 15 (carries p.Trp727Cys) seems to diverge from the majority of the cohort as this individual presents with hypertonia and no signs of hypotonia, is the only individual with notable sleep deficits, and is the only individual with a rigid gait. Also, this person does not have any known neurological features despite undergoing an EEG and MRI testing. Overall, more directed investigation is needed to better understand the different roles p.Ala546Val and p.Trp727Cys may play in different tissues and at different developmental stages.

Interestingly, proband 5's variant of p.Lys152del is only found at a 16% mosaicism by Sanger sequencing (21% by WGS) in blood. We believe that the deletion of p.Lys152 within this critical domain can explain this individual's phenotype given similarities of this person's symptoms to others in the cohort, our fly data showing that the nearby variant of p.Asp156Asn significantly impacted the function of TNPO2 in multiple tissues (see **Fig. 7C**) and the fact that this variant is at a conserved amino acid in multiple organisms including mice (see data in MARRVEL[48]). It is also important to note that the amount of mosaicism in other tissues is not known.

Except for p.Ala546Val (proband 11), and potentially p.Trp727Cys (proband 15), no obvious association with the other variants tested in the fly and symptoms or severity of individual's features are observed. We hypothesize that this is due to the findings that both up- or down-regulation of *Transportin* in the fly cause similar phenotypes (see **Fig. 2, 5,** and **S1**). It is likely that loss of *dTnpo* disrupts the shuttling of cargoes into the nucleus and this disrupts multiple pathways important during development and for neuron maintenance (see below). Speculatively, we predict that upregulation of *dTnpo* and ectopic expression of *hTNPO2* causes similar phenotypes as *dTnpo* loss as these would cause an accumulation of the Transportin protein intracellularly. This could sequester the dTnpo-cargos, making them unavailable to

perform their normal functions. Thus, gain-of-function associated toxicity would still result in similar phenotypes as loss-of-function mechanisms.

It is important to note that TNPO1/2 function in many pathways including ciliogenesis, mitotic spindle assembly, and nuclear envelope assembly. Further, it was recently shown that when TNPO1 binds the nuclear pore complex that RAN GTP was retained in the nucleus[88]. Thus, disrupting the function of TNPO2 could not only impact its cargoes but also the RAN GDP/GTP gradient used to drive the activity of this and other proteins[7]. Notably, due to this pleiotropic nature of TNPO2, the impact of up/downregulating the fly gene, the impact of ectopically expressing the human gene in the fly, and the impact of variants on the endogenous function of the protein encoded by human *TNPO2* in individuals is likely to be very complex. Interestingly, phenotypic variability of monogenic causes of neurologic disorders has been described for other genetic conditions, such as those associated with *EEF1A2* (MIM: 602959)[89]. Overall, our fly data demonstrate that Transportin's activity would likely need to be tightly regulated to prevent disease.

Supporting the hypothesis that both gain- or loss-of-function variants can contribute to *TNPO2*-associated disorder, it is notable that copy number variants (CNVs) that include the *TNPO2* gene, both deletions and duplications, have both been reported as pathogenic in ClinVar[90] by studies that evaluated CNVs in individuals with developmental delays, including accession numbers: VCV000059111.1 (SCV000080263.4; duplication)[91] and VCV000153069.1 (SCV000182485.3; deletion)[92]. While the region of these CNVs includes other genes, it is intriguing to consider that the dosage-sensitivity of *TNPO2* could contribute to developmental phenotypes in these individuals. In particular, the whole gene deletion of *TNPO2* in these cases are consistent with a potential haploinsufficiency as a part of the disease mechanism(s) associated with this gene.

In sum, our fly data support that any disruptions to *TNPO2* activity (gain or loss) in individuals is likely to cause similar symptoms. Further, coding variants in TNPO2 can alter the

function of the protein with ectopic expression of transgenes. Future mechanistic studies should focus on differences between the variants, considering the impact of variants as gain-of-function, loss-of-function, or dominant-negative mutations in multiple contexts and tissues. Further, additional variants will need to be tested to better understand the potential association between protein domain and a variant's impact on TNPO2's function. Last, as our studies depended on ectopic expression of human *TNPO2*, future studies should focus on defining the mechanisms underlying the impact of individual variants in an endogenous system.

TNPO2 during development.

Consistent with TNPO2 being a pleiotropic protein, it is required in multiple tissues in the fly (see **Fig. 2, 4, and S1**). Impacts of losing *Transportin* during development are dosage-dependent with stronger *dTnpo* mutants and *dTnpo*-targeting RNAi causing more severe defects (see **Fig. 2** and **S1**). Upregulation of *dTnpo* and ectopic expression of its human orthologue, *hTNPO2*, causes similar defects (see **Fig. 5-7**), potentially by tittering cargoes away from their normal function(s) as discussed above. We note that our *dTnpo* mutant and RNAi studies rely on robust depletion of *dTnpo*, either through the use of homozygous mutant animals or the significant downregulation of *dTnpo* mRNA, respectively (see **Fig. 2, S1**). Further, the *UAS::dTnpo*^{GS11030} allele causes a dramatic upregulation of the fly gene. Future studies should titer the expression of *dTnpo* to see when phenotypes occur and consider the possibility that heterozygous mutant animals may have minor anomalies. It is also important to note that the ectopic expression of the *UAS-hTNPO2* lines is not expected to cause such robust overexpression of the Transportin protein as was seen with the *UAS::dTnpo*^{GS11030} allele[69]. However, further investigations into the impacts of the variants in a system that does not rely on ectopic expression of genes would likely reveal additional information as to the role these variants play in disease.

Interestingly, TNPO1/2 interact with multiple, conserved factors important in developmental pathways, including NF- κB signaling[18], hedgehog signaling[60], insulin signaling[29], Ras/ERK signaling[17,93,94]. These pathways are involved in multiple aspects of fly eye and wing formation and disruptions can cause similar phenotypes to what we observe in our studies[65,67,68]. Thus, data support that TNPO2 coding variants could impact multiple developmental pathways simultaneously and this could explain the varied features observed in our cohort (see **Data S1**). Further, TNPO2 was found to be critical for HuR/ElavL1-mediated muscle cell differentiation in cultured murine myoblasts[11]. This may be related to muscle tone abnormalities in our cohort (see **Table 1**). Last, it is notable that most of the *TNPO2* cohort present with gastrointestinal abnormalities while multiple TNPO1/2 cargoes are involved in stress pathways associated with chronic intestinal inflammation in mammals, including components of the Activator Protein-1 (AP1) transcription complex[95–98], NEMO[18,99], ADAR1 (MIM: 146920)[100,101], and HSP70 (MIM: 140550)[16,102]. While gastrointestinal abnormalities are commonly associated with GDD, symptoms may be exacerbated by*TNPO2* variants. Overall, our findings in *Drosophila* support that *hTNPO2* is an important developmental gene that can impact multiple systems.

TNPO2 in the nervous system.

Accumulating data show that *hTNPO2* is an important neuronal gene. We found that *dTnpo* is primarily expressed in a subset of neurons, including MB neurons, in the fly CNS (see **Fig. 3)**. These findings are consistent with mammalian data as *TNPO1/2* is highly expressed in the brain[9,10,53]. In mouse brains, *Tnpo2* seems to be more highly expressed overall than *Tnpo1*[9,10] but this may depend on the brain region[53]. Interestingly, when considering regions associated with memory, *Tnpo2* was shown to be more highly expressed in the cerebral cortex than *Tnpo1* and both genes were highly expressed in the hippocampus and cerebellum[53]. The hippocampus is considered most homologous to the MB in flies as neurons of both are critical for associative learning and circadian rythms[74]. Fittingly, *Tnpo1/2* activity impacts circadian rhythms in

mice[53,103] and proband 15 has sleep disturbance. Attention deficit disorder (ADD) and autism spectrum disorder (ASD), which are seen in the majority of our cohort, are commonly associated with sleep deficits[104]. Further, our data show that *dTnpo* reduction in mature neurons disrupts their function and survival of the flies (see **Fig. 4**). This may be the result of many of its cargoes being important neuronal proteins including FET proteins[105] (FUS[14], EWS [MIM: 133450][106], TAF15 [MIM: 601574]), HuR/ElavL1[8,11,15,16], hnRNPA1[8,17], and Huntington (HTT [MIM: 613004])[107]. Thus, perturbations to the translocation of these and other ubiquitous cargoes into the nucleus due to disrupted TNPO1/2 activity are likely to contribute to neurotoxicity. In fact, HuR and FUS have been implicated in ASD[108,109] and FET proteins in ADD[110]. It is also notable that homozygous null *Tnpo2* mice are viable but may have significant anxiety and locomotion abnormalities[111]. Mouse phenotypes are likely weaker than fly phenotypes because of compensation from *Tnpo1* for *Tnpo2* loss.

Overall, the data support that disruptions to TNPO2 activity can contribute to GDD, intellectual disability, behavioral deficits and neurologic features observed in our cohort (see **Tables 1** and **S1**).

Potential genetic interactions.

As TNPO2 is shown to be a dosage-dependent and pleiotropic protein, an individual's unique genetic profile may contribute to disease occurrence and presentation. Interestingly, probands 5, 6 and 15 carry heterozygous variants of uncertain significance (VUS) in genes highly expressed in the brain and predicted to be involved in neurological pathways, including *CUX2* (a transcription factor involved in neuron proliferation, differentiation and synaptic plasticity[112,113]), *ANKFY1* (likely involved in vesicle trafficking[114] and required for murine brain development[115]) and *INA* (a class IV neuronal intermediate filament involved in neuron morphogenesis[116]) (see **Note S1**). Thus, there is potential for genetic interactions between heterozygous loss of these genes and *TNPO2* variants. Further, proband 8 carries a VUS in

ARCMC9 which is associated with Joubert syndrome 30 (JBTS30)[117]. Joubert syndrome is an autosomal recessive disorder which also involves GDD, ophthalmologic abnormalities, dysmorphic features, and hypotonia. The protein encoded by *ARCMC9* impacts ciliogenesis like TNPO1/2[20–22,117]. Thus, heterozygous loss of this gene could contribute to *TNPO2*-associated phenotypes. Last, proband 14 carries a *de novo* SNV in *PDE4D* which is predicted to cause a truncation and nonsense-mediated decay. Accordingly, this individual is diagnosed with acrodysostosis 2[56]. We hypothesize that the *TNPO2* variant in this individual contributes to developmental delays.

Concluding remarks.

Overall, our data show that *TNPO2*-associated disorder represents a rare genetic condition with global developmental delay and syndromic features. As both upregulation and downregulation of *Transportin* causes similar defects in the fly and coding variants may increase or decrease hTNPO2's activity, it is difficult to differentiate symptoms associated with a gain- or loss-of-function variant in individuals within this first cohort. We conclude that because of pleiotropic effects of *TNPO2* variants, sequencing and phenotypic comparison to reported cases is the most valuable approach to diagnosing features related to *TNPO2*. Further examination of these cases will likely delineate the genotype-phenotype correlation.

SUPPLEMENTARY INFORMATION

Supplementary data include one Note, two Data files, three Figures and two Tables.

DATA AND CODE AVAILABILITY

This study did not generate datasets. All reagents developed in this study are available upon request.

DECLARATION OF INTERESTS

The Department of Molecular and Human Genetics at Baylor College of Medicine receives revenue from clinical genetic testing completed at Baylor Genetics Laboratories. YS and AB are employees of GeneDx, Inc.

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WEB RESOURCES

- ClinVar[90], accessed April, 2021, World Wide Web URL: https://www.ncbi.nlm.nih.gov/clinvar
- Combined Annotation Dependent Depletion, CADD[52] (v1.4), World Wide Web URL: https://cadd.gs.washington.edu/
- Drosophila RNAi Screening Center (DRSC) Integrative Ortholog Prediction Tool, DIOPT[62] (v7.1), World Wide Web URL: https://www.flyrnai.org/cgi-bin/DRSC_orthologs.pl
- GeneMatcher[31], accessed Dec., 2020, World Wide Web URL: https://genematcher.org/
- genome Aggregation Database, gnomAD[51] (v2.1.1), World Wide Web URL: https://gnomad.broadinstitute.org/
- Model organism Aggregated Resources for Rare Variant ExpLoration, MARRVEL[48] (v2), World Wide Web URL: http://marrvel.org/
- Online Mendelian Inheritance in Man, OMIM. McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University (Baltimore, MD), update April 11, 2021. World Wide Web URL: https://www.omim.org/

FIGURE TITLES AND LEGENDS

Figure 1: TNPO2 variants are associated with varied dysmorphic features in individuals. (**A**) Proband 4 at age 3 years with short philtrum, broad nasal bridge, large fleshy ears, and coarse facial features. (**B**) Proband 8 at age 8 years with strabismus, high nasal bridge, eversion of the lower lip, and clinodactyly. (**C**) Proband 11 at age 9 years has no clear dysmorphism. (**D**) Proband 13 at age 11 years with deep set eyes and large cupped ears.

Figure 2: Fly Transportin is essential for proper animal development and *dTnpo* loss in eyes and wings causes dysmorphisms. (**A**) Protein sequence comparison of human TNPO2 (hTNPO2) and *Drosophila* Tnpo (dTnpo) shown as a diagram and a detailed amino acid alignment. All variants are at conserved amino acids (red) except p.Lys118Asn (orange). Symbols in the protein alignment: identical (|), similar (:), different (.), absent (_). (**B**) *dTnpo* mutants (red) created for loss-of-function (LOF) studies include $dTnpo^{\Delta 11}$ (an imprecise excision of the P-element, *NP4408*), $dTnpo^{Gly736Asp}$ (an EMS-induced mutation), and a *CRIMIC* allele. Two independent RNAi lines, RNAi-1 and RNAi-2, were also obtained. (**C**) Animals homozygous for *dTnpo* mutant alleles demonstrate larval lethality due to *dTnpo* loss. None of the alleles or a large deficiency allele which lacks *dTnpo*, *Df(3L)Exel8101*, complement each other. Lethality caused by $dTnpo^{\Delta 11}$ and $dTnpo^{Gly736Asp}$ can be rescued using a genomic rescue construct, GR^{dTnpo}. (**D**) The FRT/FLP system was used to make mosaic tissue in the fly eye during development. $dTnpo^{Gly736Asp}$ causes a rough eye phenotype. No homozygous $dTnpo^{\Delta 11}$ mutant tissue is observed, indicating cell lethality. Scale bar = 100μm. (**E**) The FRT/FLP system was used to make mosaic tissue in the developing wing. $dTnpo^{Gly736Asp}$ causes notch and blister phenotypes. Scale bar = 200μm. D-E “Control” is *yw;; FRT80B*. Full fly genotypes for this and following figures are in **Data S2**. *dTnpo*-targeting RNAi produce consistent phenotypes (see **Fig. S1**).

Figure 3: *dTnpo* is highly expressed in neurons, including mushroom body neurons.

The *dTnpo CRIMIC* (*T2A-GAL4*) allele was used to drive expression of *UAS-fluorescent reporter transgenes*. (**A-L**) *UAS-mCherry.NLS* (nuclear mCherry) was expressed and tissue were dissected from L3 larvae (CNS, includes central brain and VNC) or adults (brain). Shown is half of the adult brain. Tissue were counterstained with markers for neurons (Elav) or glia (Repo). Z-stacked images showing *dTnpo* expression pattern compared to neurons (**A-F**) or glia (**G-L**). Dashed squares indicate regions used in A'-L'. (**A'-L'**) Single slice images were used to better visualize cellular co-localization of mCherry.NLS signal with neurons or glia. White arrows highlight co-localized nuclei with most neurons and some glia. (**M-R**) *dTnpo CRIMIC* driven expression of *UAS-mCD8::RFP* (membrane-bound RFP) and FasII counter-staining confirmed overlap of *dTnpo* expression and mushroom body (MB) neurons in both larval and adult brains. (**S, T**) Schematics of the larval CNS (S) and adult brain (T) highlighting MB neurons (blue), the ventral nerve cord (VNC), the central brain, and optic lobes (OL). The adult OL includes the medulla and lamina. The adult brain also includes the subesophageal ganglion (not shown in the schematic).

Figure 4: Fly Transportin is required in neurons for survival and eye function.

(**A**) The drug inducible *elav-GAL4*^{GS} driver was used to express RNAi in adult fly neurons while avoiding RNAi expression during development. Expression of *dTnpo* RNAi-1 significantly impacts animal survival, indicating a progressive loss of neuron function due to *dTnpo* loss. (**B, C**) *Rh1-GAL4* was used to express RNAi in mature photoreceptor neurons and electroretinograms (ERGs) were used to measure neuronal function at 7d, 14d, and 22d. Blue annotation shows where amplitudes are measured. Orange bars indicate the light pulses. *dTnpo* RNAi-1 nearly abolishes ON and OFF transients (**D, E**) and reduces the light coincident receptor potential (LCRP; **F**) compared to a control RNAi. Statistics: (A) log-rank, (D-F) 2-way ANOVAs

with Sidak's multiple comparisons test. P-values: *<0.02, **<0.01, ***<0.001, ****<0.0001. Each dot represents the mean of 5 recorded ERGs per animal. The mean from 5-6 animals is shown. Error bars denote SEM. "Control (Ctrl) RNAi" is *UAS-Luciferase RNAi* (TRiP.JF01355). *UAS-dTnpo RNAi-1* is TRiP.HMJ23009.

Figure 5: Upregulation of *dTnpo* disrupts morphology of eyes and wings. (**A**) Ubiquitous expression of *UAS::dTnpo*^{GS11030} using *da-GAL4* in flies causes a 25-fold increase in *dTnpo* mRNA levels by qPCR. L3 larvae were analyzed at 22°C. Unpaired t-test, P-value ***=0.0003. Each dot represents the mean from replicate wells per sample. The mean from 4 individual samples is shown. Error bars denote SD. (**B**) *da-GAL4 > UAS::dTnpo*^{GS11030} animals do not survive beyond pupariation at 25°C. (**C, D**) Upregulation of *dTnpo* during eye development, using either *ey-GAL4* (early development) or *GMR-GAL4* (late development) driven expression of *UAS::dTnpo*^{GS11030}, causes small eyes and rough eye phenotypes. Scale bar = 100μm. (**E**) *nub-GAL4* driven expression of *UAS::dTnpo*^{GS11030} causes notch and blister phenotypes (arrows) in the fly wing. Scale bar = 200μm. "Control (Ctrl)" is *UAS-empty*.

Figure 6: Variants of hTNPO2 disrupt toxicity in fly development and in the eye.
(**A**) *UAS-hTNPO2* fly lines were generated. Western immunoblots (WBs) confirmed hTNPO2 protein levels are similar between lines using a drug-inducible ubiquitous driver (*da-GAL4*^{GS}) to express transgenes and a human TNPO1/2 antibody. Normalized hTNPO2 band density from three independent westerns were quantified. Each dot represents one independent sample. The mean from 3 individual samples is shown. (**B**) *da-GAL4* driven ectopic expression of *UAS-hTNPO2:WT*^{HA} reduces Mendelian ratios compared to *UAS* control flies, demonstrating toxicity during development. Variants *p.Gln28Arg* and *p.Asp156Asn* are more toxic than *hTNPO2:WT* whereas *p.Trp727Cys* is less toxic. Each dot represents one independent cross with >100 animals scored. The mean from three independent crosses is shown. (**C, D**) Ectopic expression

of *UAS-hTNPO2:WT^{HA}* disrupts eye development using either *ey-GAL4* (early development) or *GMR-GAL4* (late development). Scale bars = 100μm. (**C**) With *ey-GAL4 > hTNPO2:WT^{HA}*, eyes are smaller than controls and have a rough eye phenotype. *p.Trp370Cys* and *p.Trp370Arg* are less toxic. (**D**) With *GMR-GAL4 > hTNPO2:WT^{HA}*, eyes are moderately smaller and there is a mild rough eye phenotype compared to controls. *p.Gln28Arg* and *p.Asp156Asn* are more toxic. Statistics: 1-way ANOVAs with Dunnett's (A) or Tukey's (B) multiple comparisons test. P-values: no significance (n.s.) ≥0.05, *<0.05, **<0.01, ***<0.001, ****<0.0001. Error bars denote SD. "UAS Control" is *UAS-empty*.

Figure 7: hTNPO2 variants alter hTNPO2-induced phenotypes and penetrance in the fly wing and variant impacts associate with their location within the protein.

(**A**) Ectopic expression of *UAS-hTNPO2:WT^{HA}* using *nub-GAL4* disrupts wing development, causing notching, blisters, and gain-of-vein phenotypes (arrows). *p.Trp370Cys*, *p.Trp370Arg*, and *p.Ala546Val* have less severe phenotypes whereas *p.Gln28Arg* and *p.Asp156Asn* are significantly more toxic. *p.Trp727Cys* has a moderately stronger gain-of-vein phenotype than *hTNPO2:WT*. Scale bar = 200μm. (**B**) Blister and notch phenotypes caused by *hTNPO2* expression in the wing occurs in 50% of wings, representing penetrance. Penetrance is significantly different for all variants except *p.Trp727Cys*. Statistics: 1-way ANOVAs with Dunnett's multiple comparisons test. P-values: no significance (n.s.) ≥0.05, *<0.05, ***<0.001, ****<0.0001. Error bars denote SEM. Each dot represents the results from one cross with >50 animals scored. The mean from two independent experiments that included two-three individual crosses is shown. (A, B) "*UAS Control*" is *UAS-empty*. (**C**) Table summarizing phenotype severity associated with variants when compared to *hTNPO2:WT*-associated phenotypes. Symbols: strong decrease in toxicity (green arrows), strong increase in toxicity (red arrows), mild increase in toxicity (orange arrows), no obvious difference in toxicity (dash). *p.Trp727Cys*

strongly reduces toxicity in two situations and mildly increases toxicity in two situations, earning two green and one red arrow in the summary.

	Summary	Proband														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Protein variant (p.)	SNV, del, delins	Gln28Arg	Gln32Arg	Pro61Arg	Lys118Asn	Lys152del, mosaic (16/21%)	Asp156Asn	Trp370Arg	Trp370Cys	Lys491_Arg492delinsGlnTrp	Pro514Leu	Ala546Val	Ser548Phe	Phe598Leu	Ala649_Leu652del	Trp727Cys
CADD score	**22.7-34.0**	27.8	24.3	23.9	22.7	–	27.1	34.0	28.8	–	30.0	27.0	31.0	29.4	–	25.5
Sanger confirmed?	**10 of 15**	yes	yes	–	yes	yes	–	yes	yes	yes	–	yes	–	yes	–	yes
Inheritance	***de novo***	*de novo*	*de novo*	*de novo*	*de novo*	*de novo*	*de novo*	*de novo*	*de novo*	*de novo*	*de novo*	*de novo*	*de novo*	mother, mosaic	*de novo*	*de novo*
Additional SNV/CNV of uncertain significance	**6 of 15**	–	–	–	–	SETBP1:p.Leu1522Argfs*59; CUX2: p.His1253Pro; *12q13.13_dup*	ANKFY1: p.Thr1088Serfs*9	–	ARMC9: p.Asp330Asn	–	*1q21.1_ins522Kb*	–	–	–	PDE4D: p.Arg237*	INA p.Leu376Pro, mosaic (20%)
Age at onset	**neo-18mo**	1mo	4mo	neonat.	13mo	3mo	6mo	4mo	neonatal	prenatal	9mo	8mo	neonat.	15mo	18mo	neonat.
Age at exam	**14mo-20y**	6y	18mo	6mo	3y	23mo	4y	10y	8y	14mo	5y	9y	20y	11y	12y	7y
Global Developmental Delays	**15 of 15**	++	+, regress.	+	++, regress.	+++	++	+++	+	+	++	+	+++	+, regress.	+	++
Speech impaired	**15 of 15**	++	+	+	++	+++	+++	+++	+	+	++	+	+++	+	+	++
Intellectual disability	**9 of 9**	+++	n/d	n/d	+++	n/d	++	+++	++	n/d	n/d	++	+++	+	++	n/d
Motor Impaired	**15 of 15**	+	+	+	++	+++	++	+++	+	+	+	+	+++	+	+	+
Dysmorphic features	**11 of 15**	+	–	+	+	–	+	–	+	+	+	–	+	+	+	+
Behavioral deficits	**10 of 14**	+	+	+	+	–	+	–	+	–	–	+	n/d	+	+	+
GI / feeding abnormalities	**11 of 15**	+	+	+	–	+	–	+	+	–	+	–	+	+	+	+
Ophthalmologic abnormalities	**10 of 15**	+	–	–	+	+	+	–	+	+	+	+	–	+	–	+
Muscle tone abnormalities	**11 of 15**	+, hypo	–	+, hypo	–	+, hypo	+, variable	+, hypo	–	+, hypo	+, variable	–	+, hypo	+, hypo	+, hypo	+, hyper
Movement /neurological disorder	**6 of 15**	+	–	–	–	+	+	–	–	–	–	+	+	+	–	-
Seizures	**6 of 15**	+, febrile	–	–	–	–	+, febrile to non-febrile	+, febrile to non-febrile	–	–	+, febrile to non-febrile	+, febrile to non-febrile	+	–	–	–
Microcephaly	**5 of 15**	+	–	–	+	+	+	–	–	+	–	–	–	–	–	–
MRI brain abnormalities	**7 of 13**	–	n/d	+	–	+	+	–	+	+	+	–	+	–	n/d	–

Table 1: Individuals with *TNPO2* variants present with developmental delays, intellectual disability, behavioral deficits and strabismus. misZ for *TNPO2* loss is 5.88 (o/e = 0.28). pLI for *TNPO2* loss is 1.00 (o/e = 0.04). *TNPO2* coding DNA (NM_001136196.1). All individuals are heterozygous for variants. No variants are found in control genetic databases. See **Data S1** and **Note S1** for additional details on persons features and additional variants of uncertain significance. no data (n/d), combined annotation dependent depletion (CADD).

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